UNIVERSITÀ DEGLI STUDI DI MILANO
Department of Biosciences

Ph.D. Course in Environmental Sciences
XXX Cycle

Investigations on the detoxification mechanisms of the malaria vector *Anopheles stephensi*

Ph.D. thesis
Marco Ferrari
R11031

Scientific tutors: Prof. Claudio Bandi
Prof. Sara Epis

Academic Year: 2016-2017

ABSTRACT IN ENGLISH

This thesis is focused on the "defensome" response to a chemical stimulus in *Anopheles stephensi*, the major malaria vector in Southern Asia. Malaria is a parasitic disease caused by parasites belonging to the *Plasmodium* genus; five species of *Plasmodium* are known to be etiological agents of the disease in humans. According to the World Health Organization (WHO), malaria caused around 445 thousand deaths and 216 million cases in 2016, representing a threat to the health of populations living in endemic areas and an impediment to the development of Third World countries. Since the Fifties of the past century, eradication campaigns have been attempted to control and eradicate this disease, relying essentially on two main components: antimalaric drugs to cure infections and insecticides and repellents to prevent new cases. Unfortunately, the strong selective pressure against the parasites and the vectors (Anopheline mosquitoes) led to the insurgence, in several countries, of resistances against the major drugs and insecticides used in malaria control, threatening the health of million people and the goals posed by the WHO. To fight this phenomenon, it is necessary to understand the xenobiotic detoxification mechanisms both in the parasites and in the vectors in order to identify new targets for the development of strategies aimed to restore the effectiveness of the weapons we have nowadays.

The first paper presented in this thesis focuses on the response of the whole chemical defensome in larvae of a sensitive *An. stephensi* strain exposed to permethrin, a pyrethroid, the most used insecticide for vector control and for crop spraying. Through a comparative transcriptomic approach, it has been possible to determine the response of enzymes and membrane transporters implied in the different detoxification phases of the so called chemical defensome. Results allowed the identification of a modulated response across time of the different defensome components and several other genes not strictly connected to detoxification, highlighting a reallocation of the depleted metabolic resources in response to the action of the insecticide. This study also confirms the involvement of ATP-binding cassette (ABC) transporters in the detoxification processes against pyrethroids in *An. stephensi*.

To investigate whether the same transporters were involved in the response to the same insecticide, we also tested the adult stage of the vector. Results underline that two out of six of the analysed genes (ABCG4 and ABCBmember6) are up-regulated in a sex-dependent way that might contribute to the higher permethrin susceptibility of male mosquitoes if compared to females.

In both these works it is evident how a particular ABC transporter belonging to the G sub-family is constantly up-regulated: the ABCG4 gene. To evaluate the importance of the ABCG4 gene in *An. stephensi* larvae, a post-transcriptional silencing technique called RNA interference (RNAi) has been

applied. A down-regulation of the transporter through the use of specific small interfering RNA (siRNA) has been achieved, preventing the extrusion of the insecticide and inducing an increased larval mortality with low doses of siRNA. Furthermore, a RNAi systemic effect spreading to tissues beyond the gut of the larvae has been demonstrated, and also the persistence of the silencing all along the development to adults when exposed to the insecticide.

To characterize the ABC transporter response against different insecticide classes, in the two last articles, *An. stephensi* larvae have been exposed to the temephos insecticide, organophosphate usually applied for larval control, and to an *Azadirachta indica* seed extract. In both cases, it was not present a differential expression of the transporters when compared to a control of non-treated larvae. Also, it was not possible to achieve a mortality increase using the insecticides in combination with verapamil, an inhibitor of the ABCs. These results highlight the wide variability in the response of these transporters in larvae of *An. stephensi* based on the type of insecticide administered.

ABSTRACT IN ITALIANO

Il lavoro di tesi si è focalizzato sulla risposta del defensoma ad uno stimolo chimico in *Anopheles stephensi*, il principale vettore malarico nel sud dell'Asia. La malaria è una malattia parassitaria che ha come agenti eziologici parassiti appartenente al genere *Plasmodio*, di cui cinque specie sono note per essere responsabili per la malattia nell'uomo. Secondo l'Organizzazione Mondiale della Sanità (World Health Organization, WHO) la malaria ha causato nel 2016 circa 445.000 morti e 216 milioni di casi, rappresentando una minaccia alla salute delle popolazioni che vivono in zone endemiche ed un impedimento allo sviluppo dei Paesi del Terzo Mondo. Per cercare di controllare ed eliminare la malattia, a partire dagli anni cinquanta del novecento sono state condotte diverse campagne di eradicazione basate essenzialmente su due componenti principali: la cura dell'infezione in soggetti infetti con farmaci antimalarici e la prevenzione dell'infezione tramite l'utilizzo di insetticidi e repellenti. La forte pressione selettiva contro il parassita e contro il vettore (zanzare del genere *Anopheles*) ha però portato in questi ultimi all'insorgenza, in diverse parti del mondo, di resistenze contro i principali farmaci e insetticidi utilizzati nel controllo della malaria, minacciando la salute di milioni di persone ed il raggiungimento degli obbiettivi posti dalla WHO. Per combattere questo fenomeno si rende necessario comprendere i meccanismi di detossificazione contro gli xenobiotici sia nel parassita che nel vettore e identificare nuovi target per lo sviluppo di strategie volte a restituire efficacia alle armi di cui disponiamo nella lotta contro questa malattia.

Il primo articolo presentato in questa tesi si focalizza sulla risposta dell'intero defensoma chimico nello stadio larvale di un ceppo sensibile di *An. stephensi* in seguito all'esposizione ad un piretroide, la permetrina, insetticida maggiormente utilizzato nel controllo di insetti vettori e per l'irrorazione di coltivazioni. Attraverso l'utilizzo di un approccio di trascrittomica comparativa, è stato possibile determinare la risposta di enzimi e trasportatori di membrana implicati nelle diverse fasi della detossificazione facenti parte del così detto defensoma chimico. I risultati ottenuti hanno permesso di individuare una risposta modulata nel tempo dei diversi componenti del defensoma, ma anche di svariati altri geni non strettamente collegati alla detossificazione, evidenziando come l'esposizione all'insetticida induca una riallocazione delle risorse metaboliche deplete dall'azione dell'insetticida stesso. Questo studio conferma inoltre il coinvolgimento dei trasportatori ATP-binding cassette (ABC) nei processi di detossificazione dai piretroidi in *An. stephensi*.

Si è quindi voluto investigare se questi stessi trasportatori siano implicati nella risposta al medesimo insetticida anche allo stadio adulto del vettore. I risultati evidenziano come due dei sei geni analizzati (ABCG4 e ABCBmember6) mostrino una sovraregolazione con una risposta dipendente dal sesso degli individui di appartenenza. Questa differenza legata al sesso potrebbe contribuire a spiegare la

maggior sensibilità degli individui di sesso maschile rispetto a quelli di sesso femminile in seguito all'esposizione all'insetticida.

In entrambi questi lavori, si evidenzia in particolare come un trasportatore ABC appartenente alla sub-famiglia G sia costantemente sovraregolato: l'ABCG4. Per valutare l'importanza del gene ABCG4 in larve di *An. stephensi* si è quindi deciso di utilizzare una tecnica di silenziamento post-trascrizionale chiamata RNA interference (RNAi). Con l'utilizzo di specifici small interfering RNA (siRNA) si è indotta la sottoregolazione del trasportatore che non è stato quindi in grado di svolgere il suo ruolo nell'estrusione dell'insetticida, inducendo un aumento della mortalità larvale a basse dosi di siRNA. Dopo aver verificato la presenza di un effetto sistemico della RNAi che si propaga al di là dell'intestino della larva, si è anche dimostrato che il silenziamento effettuato nella larva permane nell'adulto esposto all'insetticida.

Al fine di caratterizzare la risposta dei trasportatori ABC contro diverse classi di insetticidi, negli ultimi due articoli presentati si sono esposte larve di *An. stephensi* all'insetticida temephos, organofosfato generalmente utilizzato per il controllo larvale, e ad un estratto di semi di *Azadirachta indica*. In entrambi i casi, non si è evidenziata un'espressione differenziale rispetto a larve non trattate, né si è ottenuto un aumento di mortalità somministrando, oltre agli insetticidi, anche un inibitore dei trasportatori ABC, il verapamil. Si è quindi evidenziato come, in larve di *An. stephensi*, la risposta di questi trasportatori di membrana sia ampiamente variabile in base al tipo di insetticida utilizzato.

INDEX

INTRODUCTION

Malaria

Malaria is a parasitic disease caused by protozoans belonging to the Plasmodium genus. More than 150 species of Plasmodium exist in the wild affecting different vertebrate species including several primates, rodents, lizards and birds, but only four species are etiological agents of human malaria: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. Another species, *P. knowlesi*, naturally affects long-tailed and pig-tailed macaques, but it is transmitted to humans as well, representing a case of zoonotic malaria (Singh & Daneshvar, 2013).

In these parasites humans could be seen as the intermediate host where the asexual cycle is carried on in the liver and blood from the sporozoitc to the gametocytic stage. Gametocytes are then ingested by female mosquitoes belonging to the genus *Anopheles,* where the sexual cycle is completed. In the midgut of the vector mosquitoes, microgametes penetrate macrogametes to form zygotes developing into ookinetes that invade the wall of the midgut and transform into oocyst that release sporozoites. At this stage, the parasites move to the salivary glands of the mosquitoes to be injected again in the human host. Around forty species of *Anopheles* mosquitoes are competent malaria vectors due to eco-ethological factors: abundance, longevity, capacity and anthropophily (Paul et al., 2004; Mueller et al., 2010). The geographical distribution of malaria is therefore strictly connected to the presence of the Anopheline mosquitoes, essentials for its transmission. Indeed, the abundance of the vector is important to guarantee that individuals encounter and feed on infected humans and the mosquito must live enough to bite another host to transmit the parasite. Also, the salivary gland capacity of the vector is a key component for malaria spread because glands must contain a sufficient number of parasites to start a successful infection in the intermediate host that must be preferentially a human.

Medical and economic impact of malaria and Eradication programmes

Sachs and colleagues (2004) identified a high disease burden as one of the five factors responsible for the persistency of the “poverty trap” in third world countries. The term “poverty trap” indicates a self-reinforcing mechanism responsible for the persistence of poverty. They underline that Tropical sub-Saharan Africa, one of the poorest areas in the world, is also one of the areas with higher prevalence of several diseases and with numerous endemic tropical diseases. The high rates of morbidity and mortality could be reduced with higher resource investment, but those countries can’t afford such expenses with consequent higher healthcare system costs, reduction in productivity due to days off-work and foreign investment reduction frustrated by malaria impact. Diseases are responsible of poverty not only at a national level, but also at a personal-family level forcing people to spend money for healthcare expenses to cure themselves or family members instead of saving money for improving life conditions.

The only way to improve third world countries economy and defeat malaria is through international efforts and investments. For these reasons, the World Health Organization promoted and led the

Global Malaria Eradication Program (GMEP) from 1955 to 1969 with the elimination of the disease from several areas thanks to the massive use of DDT. The program failed to accomplish its goal due to the economic crisis in the Seventies and to the overconfidence of politics assuming that all the knowledge needed for achieving eradication was already available (Najéra et al., 2011). In 1998 the Roll Back Malaria (RBM) partnership was launched involving UN agencies as UNDP and UNICEF, the World Bank, several organizations and research institutions with the aim of reducing by half the number of malaria deaths by 2010. During these 17 years malaria burden was significantly reduced, averting over 6 million deaths, mainly between children under five years old. In 2001-2013 period, anti-malarial interventions have been responsible for a reduction of 47% in the global mortality rate and a reduction of the disease incidence by 30%. Despite these results, malaria is still a major burden in the world threating more than three billion people in 97 countries and territories, with 200 million new cases and more than 400 thousand deaths worldwide in 2015 (WHO, 2016). That year, the Global Technical Strategy for Malaria 2016-2030 was developed under the leadership of the WHO Global Malaria Programme, aiming to a global mortality and incidence reduction of this disease by 90% for 2030, elimination of malaria from at least 35 countries in which it is present nowadays and prevention of new cases in malaria-free countries. (WHO, 2017). Unfortunately, these goals and results are threatened by political instability, infrastructure and organization inefficiency, climate changes and, most importantly, by the insurgence and spread of insecticide resistant vector mosquito populations and drug resistant parasites.

Vector control and insecticide resistance

Some malaria control programs are based on the reduction and prevention of infections through the control of vector mosquito populations and on the treatment of infections through artemisinin-based combination therapies (ACTs). In those areas where people do not beneficiate of healthcare systems, vector control is the only way to interrupt the diffusion of malaria. This goal is achieved through an integrated mosquito management (IMM): chemical control with the use of insecticides; mechanical control with oil and surface films to prevent mosquitoes to lay eggs and avoid larvae emergence; genetic control based on sterile insect techniques (SITs) and population replacement; biological control using toxins produced by bacteria as *Bacillus thuringiensis israelensis* and *B. sphericus*, introducing predators like fishes (e.g. *Gambusia affinis* and *Poecilia reticulata*), using insect growth regulators (IGR); personal protection through the use of Insecticide Treated Bed-nets (ITN), internal residual spraying (IRS) and repellents; environmental management reducing the number of breeding sites with a correct management of vegetation and water bodies. For an effective IMM not only all the appropriate methods must be applied, but also a knowledge of the biology of the vector is needed. Also knowing which *Anopheles* species, among the 400 existing, are vector is important to address the control efforts preventing ecological damages due to species loss and waste of resources that could be used in a better way in countries with money shortages. Many of

these techniques, however, present side effects or complications. For example, the introduction of *Gambusia affinis* for biological control led to the alteration of local ecosystems when fishes found alternative food sources to mosquito larvae, in particular other arthropods and eggs of fishes that could have been mosquito predator themselves (Pyke, 2008). For this reason, WHO does not recommend anymore the indiscriminate use of *G. affinis*, preferring to identify other local species that could play a similar predator role. Also, physical methods as the use of surface oils and films, very effective in preventing adult mosquito emergence, show off-target effect on other surface-breathing arthropods.

After the extensive use of organochlorines as DDT during the 1950s eradication campaigns, new synthetic and less toxic insecticides have been introduced for mosquito control. Nowadays the most used class of chemicals are pyrethroids, in particular permethrin, for their high effectiveness and low toxicity for mammals (Zaim et al., 2000). In particular, pyrethroids demonstrated to be essential for the treatment of bed-nets (Alout et al., 2017), that are the main line of defence against mosquito bites and malaria infection. But the continuous selection pressure due to the exposure to insecticides, like DDT, led to the accumulation of resistance genes on one side, leading to modifications of the site of action of the insecticide, and of detoxification metabolic pathways on the other side. For the resistance to pyrethroids two point mutations in a single voltage-gated sodium channel gene, L1014, are known in *Anopheles gambiae* (Martinez-Torres et al., 1998; Ranson et al., 2000), while one single mutation on the Rdl gene A302S and one on the ace-1 gene conferring resistance to dieldrin (Du et al., 2005) and carbamates and organophosphates (Weill et al., 2003) respectively. The metabolic resistance involves, instead, the biochemical transformation of the toxicant decreasing its ability to interact with the target. This is done by the amplification, overexpression and coding sequence variation in groups of genes encoding proteins and enzymes involved in detoxification processes, in particular cytochrome P450, esterases and glutathione-S-transferases (Li et al., 2007). Furthermore, the insurgence of a resistance against a specific compound can mine the effectiveness of others, in a mechanism known as cross-resistance. This mechanism is particularly dangerous for vector eradication and control programs since it is known that DDT resistance led to a decreased effectiveness of pyrethroids in *Aedes aegypti* (Brenques et al., 2003; Flores et al., 2013), *An. gambiae* (Chandre et al., 1999; Mitchell et al., 2012), *An. stephensi* (Davari et al., 2007), *Anopheles funestus* (Mulamba et al., 2014), *Anopheles arabiensis* (Nardini et al., 2013).

Detoxification mechanism and ATP-Binding-Cassette transporters

The detoxification process of a xenobiotic compound, such as insecticides, involves the biotransformation and extrusion of toxic compounds and their catabolites. It can be divided in four phases, 0-III, in which different cellular processes are involved. In phase I the xenobiotic is oxidized, reduced or hydrolysed by several enzymes such as cytochromes P450 (CYPs) and

carboxylesterases (CCEs). During phase II transferase enzymes as UDP-glucoronosyltransferases (UGTs) and glutathione S-transferases (GSTs) conjugate the product of the previous oxidation with glutathione, and other hydrophilic molecules. Both in phase 0 and III there is the activation of ATP Binding Cassette (ABC) transporters: in phase 0 their role is pumping outside of the cell the toxicant to keep the intracellular concentration low, allowing other enzymes to degrade what is not extruded; in phase III they pump out the products of detoxifying enzymes during phase I and II (Despres et al., 2007; Kennedy & Tierney, 2013). The sequence of cellular events occurring during insecticide exposure has not been clarified yet. Transcriptomic studies focus on single time-points highlighting a response of genes that are up- or down-regulated in that specific time-point (Abdalia et al., 2008; Niu et al., 2012; Faucon et al., 2015). However, this limited information makes impossible to understand how the response of these genes is modulated during time. Other studies, instead, investigate expression profiles of single gene families at different time-points. Multiple P450 genes show to be up-regulated in resistant strains of *Culex quinquefasciatus* during a time course of 12, 24, 48 and 72 hours of permethrin exposure (Liu et al., 2011; Gong et al., 2013). Other gene families show a modulated transcriptional response during time. This is the case for Cuticular Protein and ABC transporter genes in sensitive strains of *Culex pipiens pallens* (Fang et al., 2015) and *Anopheles stephensi* (Epis et al., 2014b) respectively.

ABC transporters are widely distributed through all the kingdoms of life, from Archea to Mammals, indicating an essential role for the living organisms. ABC transporters are primary active-transporters formed by two cytosolic and two integral transmembrane domains and are responsible for the transport of substrates across the cellular membranes. For their activation, binding and hydrolysis of ATP is required on the cytosolic domains. They mediate the transport of several compounds from the cytoplasm to the outside of the cell: mono and polysaccharides, amino-acids, lipids, ions, xenobiotic compounds like insecticides and drugs. For this ability to extrude xenobiotics, they have been identified as important players in insecticide and multidrug resistance in insects and human cancer cells respectively.

ABCs have been catalogued in eight subfamilies, from A to H, according similarity into the sequence of the cytosolic domain. Among these, P-glycoproteins members of the B subfamily, multidrug resistance associated proteins belonging to the C subfamily, and breast cancer cell proteins belonging to G subfamily play a significant role in multidrug resistance in humans (Dermauw & Van Leeuwen, 2014). ABC transporters are also involved in the extrusion of insecticides in several arthropod species such as *Culex pipiens* (Buss et al., 2002), the cotton bollworm *Helicoverpa armigera* (Srinivas et al., 2004), *Aedes caspius* (Porretta et al., 2008), the human body louse *Pediculus humanus humanus* (Yoon et al., 2011), *Drosophila melanogaster* (Strycharz et al., 2013) and *Anopheles stephensi* (Epis et al., 2014a, b). A member of the H sub-family has been demonstrated to be involved also in the defence against the Cry toxin of *Bacillus turingiensis* in the diamondback moth *Plutella xylostella* (Guo et al., 2015) showing the wide spectrum of action of these

proteins not only against synthetic compounds. Furthermore, the involvement of ABC transporters against at least 27 insecticides or acaricides, including DDT and pyrethroids, belonging to nine different classes has been demonstrated (Buss & Callaghan, 2008; Dermauw & Van Leeuwen, 2014). In particular, B, C and G sub-families have been reported in several studies to be involved in different species, indicating an analogue function in detoxification like in human transporters. Members of the B sub-family are linked to resistance in the Dengue vector *Ae. aegypti* (Bariami et al., 2012) and in the tobacco budworm *Heliotis virescens* (Lanning et al., 1996), while members of the C and G sub-families are over-transcribed in resistant strains of *D. melanogaster* (Jones et al., 2012; Strycharz et al., 2013) and *Bemisia tabaci* (Yang et al., 2013).
For their importance in insecticide detoxification, ABC transporters have been indicated as potential targets for silencing in order to replace chemicals or increase their effectiveness (Broehan et al., 2013; Figueira-Mansur et al., 2013).

RNA interference as strategy for vector control

Since its discovery, RNA interference (RNAi) mechanisms has been used to study gene function in several species of medical and agronomic interest (Pillai et al., 2017; Saurabh et al., 2014), in cancer research (Xu et al., 2017), for drug discovery (DeVincenzo, 2012). RNAi is a molecular process based on short interfering RNA (siRNA) sequences targeting complementary mRNA and inducing its cleavage or silencing. The process was discovered in 1998 by Fire and Mello that, in 2006, shared a Nobel Prize for their work on *Caenorhabditis elegans*. The mechanism of RNAi is pretty simple since a double stranded RNA enters in the cell and it is bound by a protein called Dicer that cut the dsRNA into siRNAs. Then these fragments form a multiprotein complex called RNA-induced silencing complex (RISC) that separate the two RNA strands and uses the complementary one to bind and cleave the mRNA target. Also, it is well known in *C. elegans* and several other species (Pillai et al., 2017) the existence of a systemic RNAi, with the interference effect not limited to a single cell but with a spreading signal to close cells. This passage from cell to cell requires a transmembrane transporter called SID-1, lacking in diptera like mosquitoes (Huvenne & Smagghe 2010). However, several studies on *Culex pipiens* (Lopez-Martinez et al., 2012), *Ae. aegypti* (Coy et al., 2012: Singh et al., 2013; Whyard et al., 2015; Dalla Bona et al., 2016) and *An. gambiae* (Zhang et al., 2010) suggest the existence of a SID-1 independent systemic RNAi pathway. In particular, the studies of Whyard et al. (2015) and Dalla Bona et al. (2016) demonstrated that the up-take of dsRNA through oral feeding in *Ae. aegypti* larvae induce a down-regulation of the target gene that persist through the development to adults. Furthermore, Dalla Bona and colleagues successfully silenced VGCS gene in a deltamethrin resistant strain of *Ae. aegypti* restoring the susceptibility to this insecticide. These results are very important to highlight the possibility of a RNAi approach to fight the problem of insecticide resistance down-regulating those genes involved in xenobiotic detoxification. Another study by Figueira-Mansur and colleagues (2013) shows that silencing a P-

glycoprotein in *Ae. aegypti* larvae increases mortality when they are exposed to temephos. Anyway, despite the promising applications of this technology, a major problem still persists for its use on the field for vector control: dsRNA delivery to mosquito larvae in the wild. In fact, the main constrains are the amount of dsRNA needed to effectively induce a down-regulation and the stability of the molecules that are exposed to nucleases and pH alterations in the environment. Some studies tried to face this problem delivering dsRNA using chitosan, carbon quantum dot and silica nanoparticles to reduce degradation and increase the concentration of molecule (Zhang et al., 2010; Das et al., 2015; Kumar et al., 2016).

Despite these problems, RNAi based vector control could be a reliable strategy to face the insurgence of insecticide resistance, both using RNAi to down-regulate resistance genes and to target genes involved in essential metabolic pathways. These approaches could have a significant impact on the amount of insecticides spread in the environment, helping to reduce pollution derived from these chemicals and avoiding off-target effects on other organisms including humans. RNAi is indeed an eco-friendly method without any risk for other species because of its intrinsic specificity for the target, necessary for an effective down-regulation.

Introduction to papers and manuscripts

In this thesis, I have included the published papers and the manuscripts that are the product of my research during the three years as a PhD student at the Veterinary and Bioscience Departments at the University of Milan. The papers can be subdivided in two main groups:

1. The former three are focused on the response of the *An. stephensi* defensome against the permethrin insecticide through comparative transcriptomic, gene expression and gene silencing approaches.

2. The latter two aimed to investigate the role of ABC transporters in the response to different insecticides to evaluate possible differences in their involvement against other chemicals.

References

Abdalla H., Matambo T.S., Koekemoer L.L., Mnzava A.P., Hunt R.H., Coetzee M. Insecticide susceptibility and vector status of natural populations of *Anopheles arabiensis* from Sudan. Trans Roy Soc Trop Med Hyg 2008, 102:263

Alout H., Labbé P., Chandre F., Cohuet A. Malaria vector control still matters despite insecticide resistance. Trends Parasitol 2017, 33(8):610

Bariami V., Jones C.M., Poupardin R., Vontas J., Ranson H. Gene amplification, ABC transporters and cytochrome P450s: unraveling the molecular basis of pyrethroid resistance in the dengue vector, *Aedes aegypti*. PLoS Negl Trop Dis 2012, 6:1692

Brengues C., Hawkes N.J., Chandre F., McCarroll L., Duchon S., Guillet P., Manguin S., Morgan J.C., Hemingway J. Pyrethroid and DDT cross-resistance in *Aedes aegypti* is correlated with novel mutations in the voltage-gated sodium channel gene. Med Vet Entomol 2003, 17(1):87

Broehan G., Kroeger T., Lorenzen M., Merzendorfer H. Functional analysis of the ATP-binding cassette (ABC) transporter gene family of *Tribolium castaneum*. BMC Genomics 2013, 14:6

Buss D.S., Callaghan A. Interaction of pesticides with p-glycoprotein and other ABC proteins: a survey of the possible importance to insecticide, herbicide and fungicide resistance. Pestic Biochem Physiol 2008, 90:141

Buss, D.S., McCaffery A.R., Callaghan A. Evidence for P-glyco-protein modification of insecticide toxicity in mosquitoes of the *Culex pipiens* complex. Med Vet Entomol 2002, 16:218

Chandre F., Darriet F., Manguin S., Brengues C., Carnevale P., Guillet P. Pyrethroid cross resistance spectrum among populations of *Anopheles gambiae* s.s. from Cote d'Ivoire. Journal of the American Mosquito Control Association 1999, 15(1):53

Coy M.R., Sanscrainte N.D., Chalaire K.C., Inberg A., Maayan I., Glick E., Paldi N., Becnel J.J. Gene silencing in adult *Aedes aegypti* mosquitoes through oral delivery of double-stranded RNA. J Appl Entomol 2012, 136:741

Dalla Bona A.C., Faitta Chitolina R., Lopes Fermino M., deCastro Poncio L., Weiss A., Pereira Lima J.B., Paldi N., Soares Bernardes E., Henen J., Maori E. Larval application of sodium channel homologous dsRNA restores pyrethroid insecticide susceptibility in a resistant adult mosquito

population. Parasit Vectors 2016, 9:397

Das S., Debnath N., Cui Y., Unrine J., Reddy Palli S. Chitosan, carbon quantum dot, and silica nanoparticle mediated dsRNA delivery for gene silencing in *Aedes aegypti*: a comparative analysis. ACS Appl Mater Interfaces 2015, 7:19530

Davari B., Vatandoost H., Oshaghi M.A., Ladonni H., Enayati A.A., Shaeghi M., Basseri H.R., Rassi Y., Hanafi-Bojd A.A. Selection of *Anopheles stephensi* with DDT and dieldrin and cross-resistance spectrum to pyrethroids and fipronil. Pestic Biochem Physiol 2007, 89(2):97

Dermauw W., Van Leeuwen T. The ABC gene family in arthropods: Comparative genomics and role in insecticide transport and resistance. Insect Biochem Mol Biol 2014, 45:89

Despres, L., David, J., Gallet, C. The evolutionary ecology of insect resistance to plant chemicals. Trends Ecol Evol 2007, 22(6):298

DeVincenzo J.P. The promise, pitfalls and progress of RNA-interference-based antiviral therapy for respiratory viruses. Antivir Ther 2012, 17(1):213

Du W., Awolola T.S., Howell P., Koekemoer L.L., Brooke B.D., Benedict M.Q., Coetzee M., Zheng L. Independent mutations in the Rdl locus confer dieldrin resistance to *Anopheles gambiae* and *An. Arabiensis*. Insect Mol Biol 2005, 14(2):179

Epis S., Porretta D., Mastrantonio V., Comandatore F., Sassera D., Rossi P., Cafarchia C., Otranto D., Favia G., Genchi C., Bandi C., Urbanelli S. ABC transporters are involved in defense against permethrin insecticide in the malaria vector *Anopheles stephensi*. Parasit Vectors 2014a, 7:349

Epis S., Porretta D., Mastrantonio V., Urbanelli S., Sassera D., De Marco L., Mereghetti V., Montagna M., Ricci I., Favia G., Bandi C. Temporal dynamics of the ABC transporter response to insecticide treatment: insights from the malaria vector *Anopheles stephensi*. Sci Rep 2014b, 4:7435

Fang F., Wang W., Zhang D., Ly Y., Zhou D., Ma L., Shen B., Sun Y., Zhu C. The cuticle proteins: a putative role for deltamethrin resistance in *Culex pipiens pallens*. Parasitol Res 2015, 114:4421

Faucon F., Dusfour I., Gaude T., Navratil V., Boyer F., Chandre F., Sirisopa P., Thanispong K., Juntarajumnong W., Poupardin R., Chareonviriyaphap T., Girod R., Corbel V., Reynaud S., David J.P. Identifying genomic changes associated with insecticide resistance in the dengue mosquito *Aedes aegypti* by deep targeted sequencing. Genome Res 2015, 25(9):1347

Figueira-Mansur J., Ferreira-Pereira A., Mansur J.F., Franco T.A., Alvarenga E.S.L., Sorgine M.H.F., Sorgine B.C., Neves B.C., Melo A.C.A., Leal W.S., Masuda H., Moreira M.F. Silencing of P-glycoprotein increases mortality in temephos-treated *Aedes aegypti* larvae. Insect Molecular Biology 2013, 22(6):648

Flores A.E., Ponce G., Silva B.G., Gutierrez S.M., Bobadilla C., Lopez B., Mercado R., Black W.C.IV. Wide spread cross resistance to pyrethroids in *Aedes aegypti* (L.) from Veracruz State Mexico. J Econ Entomol 2013, 106(2):959

Gong Y., Li T., Gao X., Liu N. Permethrin induction of multiple cutochrome P450 genes in insecticide resistant mosquitoes, *Culex quinquefasciatus*. Int J Biol Sci 2013, 9:863

Guo Z., Kang S., Zhu X., Xia J., Wu Q., Wang S., Xie W., Zhang Y. The novel ABC transporter ABCH1 is a potential target for RNAi-based insect pest control and resistance management. Sci Rep 2015, 5:13728

Huvenne H., Smagghe G. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. J Insect Physiol 2010, 56(3):227

Jones C.M., Toe H.K., Sanou A., Namountougou M., Hughes A., Diabate A., Dabire R., Simard F., Ranson H. Additional selection for insecticide resistance in urban malaria vectors: DDT Resistance in *Anopheles arabiensis* from Bobo-Dioulasso, Burkina Faso. PLoS ONE 2012, 9:e45995

Kennedy C., Tierney K. Xenobiotic protection/resistance mechanisms in organisms. In: Laws, E.A. (Ed.), Environ. Toxicol. Springer, New York 2013, pp. 689-721.

Kumar D.R., Saravana Kumar P., Rajiv Gandhi M., Al-Dhabi N.A., Paulraj M.G., Ignacimuthu S. Delivery of chitosan/dsRNA nanoparticles for silencing of wing development vestigial (vg) gene in *Aedes aegypti* mosquitoes. Int J Biol Macromol 2016, 86:89

Lanning C.L., Fine R.L., Corcoran J.J., Ayad H.M., Rose R.L., Abou-Donia M.B. Tobacco budworm P-glycoprotein: biochemical characterization and its involvement in pesticide resistance. Biochim Biophys Acta 1996, 1291(2):155

Li X., Schuler M.A., Berenbaum M.R. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Annu Rev Entomol 2007, 52:231

Liu N., Li T., Reid W.R, Yang T., Zhang L. Multiple cytochrome P450 genes: their constitutive overexpression and permethrin induction in insecticide resistant mosquitoes, *Culex quinquefasciatus*. PLoS ONE 2011, 6:e23403

Lopez-Martinez G., Meuti M., Denlinger D.L. Rehydration driven RNAi: a novel approach for effectively delivering dsRNA to mosquito larvae. J Med Entomol 2012, 49:215

Martinez-Torres D., Chandre F., Williamson M.S., Darriet F., Bergé J.B., Devonshire A.L., Guillet P., Pasteur N., Pauron D. Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae* s.s. Insec Mol Biol 1998, 7(2):179

Mitchell S.N., Stevenson B.J., Muller P., Wilding C.S., Egyir-Yawson A., Field S.G., Hemingway J., Paine M.J.I., Ranson H., Donnelly M.J. Identification and validation of a gene causing cross-resistance between insecticide classes in *Anopheles gambiae* from Ghana. Proc Natl Acad Sci U.S.A. 2012, 109(16): 6147

Mueller A.K., Kohlhepp F., Hammerschmidt C., Michel K. Invasion of mosquito salivary glands by malaria parasites: prerequisites and defense strategies. Int J Parasitol 2010, 40(11):1229

Mulamba C., Riveron J.M., Ibrahim S.S., Irving H., Barnes K.G., Mukawaya L.G., Birungi J., Wondji C.S. Widespread pyrethroid and DDT resistance in the major malaria Vector *Anopheles funestus* in East Africa is driven by metabolic resistance mechanisms. PLoS One 2014, 9(10):e110058

Najéra J.A., Gonzalez-Silva M., Alonso P. Some lessons for the future from the Global Malaria Eradication Programme (1955-1969). PLos Med 2011, 8(1):e1000412

Nardini L. Christian R.N., Coetzer N., Koekemoer L.L. DDT and pyrethroid resistance in *Anopheles arabiensis* from South Africa. Parasit Vectors 2013, 6:229

Niu J.Z., Dou W., Ding T.B., Shen G.M., Zhang K., Smagghe G., Wang J.J. Transcriptome analysis of the citrus red mite, *Panonychus citri*, and its gene expression by exposure to insecticide/acaricide. Insect Mol Biol 2012, 21(4):422

Paul R.E.L., Diallo M., Brey P.T. Mosquitoes and transmission of malaria parasites – not just vectors. Malar J 2004, 3:39

Pillai B.A., Nagarajan U., Mitra A., Krishnan U., Rajendran S., Hoti S.L., Mishra R.K. RNA interference in mosquito: understanding immune response, double-stranded RNA delivery systems and potential applications in vector control. Insect Mol Biol 2017, 26(2):127

Porretta D., Gargani M., Bellini R., Medici A., Punelli F., Urbanelli S. Defense mechanisms against insecticides temephos and diflubenzuron in the mosquito *Aedes caspius*: The P-glycoprotein efflux pumps. Med Vet Entomol 2008, 22:48

Pyke H.G. Plague minnow or mosquito fish? A review of the biology and impacts of introduced *Gambusia* species. Annu Rev Ecol Evol Syst 2008, 39:171

Ranson H., Jensen B., Vulule J.M., Wang X., Hemingway J., Collins F.H. Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. Insect Mol Biol 2000, 9(5):491

Sachs J., McArthur J.W., Schmidt-Traub G., Kruk M., Bahadur C., Faye M., McCord G. Ending Africa's Poverty Trap. Brooking Pap Econ Activity 2004, 1:117

Saurabh S., Vidyarthi A.S., Prasad D. RNA interference: concept to reality in crop improvement. Planta 2014, 239(3):543

Singh A.D., Wong S., Ryan C.P., Whyard S. Oral delivery of double-stranded RNA in larvae of the yellow fever mosquito, *Aedes aegypti*: implications for pest mosquito control. J Insect Sci 2013, 13:69

Singh B., Daneshvar C. Human Infections and detection of *Plasmodium knowlesi*. Clin Microbiol Rev 2013, 26(2):165

Srinivas R., Udikeri S. S., Jayalakshmi S. K., Sreeramulu K. Identification of factors responsible for insecticide resistance in *Helicoverpa armigera*. Comp Biochem Physiol C Toxicol Pharmacol 2004, 137(2):261

Strycharz J.P., Lao A., Li H., Qiu X., Lee S.H., Sun W., Yoon K.S., Doherty J.J., Pittendrigh B.R., Clark J.M. Resistance in the highly DDT-resistant 91-R strain of *Drosophila melanogaster* involves decreased penetration, increased metabolism, and direct excretion. Pestic Biochem Physiol 2013, 107:207

Tomoyasu Y., Miller S.C., Tomita S., Schoppmeier M., Grossmann D., Bucher G. Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in *Tribolium*. Genome Biol 2008, 9(1):R10

Weill M., Lutfalla G., Mogensen K., Chandre F., Berthomieu A., Berticat C., Pasteur N., Philips A., Fort P., Raumond M. Comparative genomics: Insecticide resistance in mosquito vectors. Nature 2003, 423(6936):136

WHO. World Malaria Report 2016. Geneva: World Health Organization, 2016

WHO. Global Technical Strategy For Malaria 2016-2030. Geneva: World Health Organization, 2017

Whyard S., Erdelyan C.N., Partridge A.L., Singh A.D., Beebe N.W., Capina R. Silencing the buzz: a new approach to population suppression of mosquitoes by feeding larvae double-stranded RNAs. Parasit Vectors 2015, 8:96

Xu X., Saw P.E., Tao W., Li Y., Ji X., Yu M., Mahmoudi M., Rasmussen J., Ayyash D., Zhou Y., Farokzad O.C., Shi J. Tumor microenvironment-responsive multistaged nanoplatform for systemic RNAi and cancer therapy. Nano Lett 2017, 17(7):4427

Yang N., Xie W., Jones C.M., Bass C., Jiao X., Yang X., Liu B., Li R., Zhang Y. Transcriptome profiling of the whitefly *Bemisia tabaci* reveals stage-specific gene expression signatures for thiamethoxam resistance. Insect Mol Biol 2013, 22:485

Yoon K.S., Strycharz J.P., Baek J.H., Sun W., Kim J.H., Kang J.S., Pittendrigh B.R., Lee S.H., Clark J.M. Brief exposures of human body lice to sublethal amounts of ivermectin over-transcribes detoxification genes involved in tolerance. Insect Mol Biol 2011, 6:687

Zaim M., Aito A., Nakashima N. Safety of pyrethroid-treated nets. Med Vet Entomol 2000, 14:1

Zhang X., Zhang J., Zhu Y. Chitosan/double-stranded RNA nanoparticle-mediated RNA interference to silence chitin synthase genes through larval feeding in the African malaria mosquito (*Anopheles gambiae*). Insect Mol Biol 2010, 19(5):683

PAPER 1:

The choreography of the chemical defensome response to insecticide stress: insights into the *Anopheles stephensi* transcriptome using RNA-Seq

SCIENTIFIC REPORTS 2017; 7:41312
DOI:10.1038/srep41312

The choreography of the chemical defensome response to insecticide stress: insights into the *Anopheles stephensi* transcriptome using RNA-Seq

Leone De Marco[1,2], Davide Sassera[1], Sara Epis[3,4], Valentina Mastrantonio[5], Marco Ferrari[3], Irene Ricci[2], Francesco Comandatore[3,4], Claudio Bandi[3], Daniele Porretta[5] & Sandra Urbanelli[5]

Author Affiliations

[1]Department of Biology and Biotechnology, University of Pavia, Pavia, Italy
[2]School of Bioscience and Veterinary Medicine, University of Camerino, Camerino, Italy
[3]Department of Biosciences, University of Milan, Milan, Italy
[4]Department of Veterinary Medicine, University of Milan, Milan, Italy
[5]Department of Environmental Biology, Sapienza, University of Rome, Rome, Italy

Received: 11 August 2016; Accepted: 19 December 2016; Published: 23 January 2017

Correspondence and requests for materials should be addressed to D.P. (email: daniele.porretta@uniroma1.it)

Acknowledgements

The authors are grateful to Dr. Paolo Rossi, Veronica Rovazzani, Tommaso Sturmo and Alessandra Spanò for providing assistance to accomplish this research. Massimo Reverberi and Alessandro Grottoli for their comments and suggestions. This work was supported by MIUR Prin 2010–2011 (to C. Genchi).

Abstract

Animals respond to chemical stress with an array of gene families and pathways termed “chemical defensome”. In arthropods, despite many defensome genes have been detected, how their activation is arranged during toxic exposure remains poorly understood. Here, we sequenced the transcriptome of *Anopheles stephensi* larvae exposed for six, 24 and 48 hours to the LD_{50} dose of the insecticide permethrin to monitor transcriptional changes of defensome genes across time. A total of 177 genes involved in insecticide defence were differentially expressed (DE) in at least one time-point, including genes encoding for Phase 0, I, II, III and antioxidant enzymes and for Heat Shock and Cuticular Proteins. Three major patterns emerged throughout time. First, most of DE genes were down-regulated at all time-points, suggesting a reallocation of energetic resources during insecticide stress. Second, single genes and clusters of genes turn off and on from six to 48 hours of treatment, showing a modulated response across time. Third, the number of up-regulated genes peaked at six hours and then decreased during exposure. Our results give a first picture of how defensome gene families respond against toxicants and provide a valuable resource for understanding how defensome genes work together during insecticide stress.

1. Introduction

A major challenge for animals is to maintain homeostasis when exposed to chemical stressors, such as endoge- nous toxic chemicals or natural and synthetic xenobiotic compounds. These toxicants have likely acted as selective factors for the evolution of an array of gene families and pathways termed chemical defensome[1], that allows an organism to sense, transform, and eliminate toxic chemicals. Comparative genomic analyses revealed the genetic redundancy and evolutionary conservation among metazoans of these gene families, that may constitute up to 2–3% of the total genome content[2,3].

Central in the chemical defensome is the biotransformation system, which comprises genes encoding for several classes of proteins that modify the toxic compound making it harmless (Detoxifying Metabolic Enzymes, DMEs). Two main phases of this detoxification process have been recognized. Phase I is characterized by the oxidation, reduction, or hydrolysis of the toxic compounds by oxidative enzymes such as cytochromes P450 (CYPs), reductive enzymes such as aldo-ketoreductases (AKRs), or epoxide hidrolases (EHs) and by the activity of carboxylesterases enzymes (CCEs)[1–6]. Phase II metabolism involves conjugation, mostly of the already oxidized chemicals, with cellular glutathione, glucuronide, or other small hydrophilic molecules by transferase enzymes as glutathione S-tansferases (GSTs), sulfotransferases (SULT), UDP-glucuronosyltransferases (UGTs)[3,7,8]. Since the 1990s, the contribute of efflux pumps to detoxification, in concert with the biotransformation system, has emerged. Detoxifying efflux pumps are proteins located in the cellular membrane, belonging to the ATP-binding Cassette transporter family (ABC transporters). ABC transporters added two additional, essential steps to the defence mechanisms against xenobiotics (Phase 0 and Phase III)[3,9]. In Phase 0, ABC-transporters actively reduce the intracellular concentration of toxicants by preventing their entry into cells or by pumping them outside the cell once they entered, while in Phase III, they expel out of the cell the toxicants that were modified by detoxifying enzymes during Phases I and II. Finally, it is now generally acknowledged that the chemical defensome is comprehensive, along with the biotransformation system, of genes encoding for antioxidant enzymes that protect cells against reactive oxygen species (ROS) generated during biotransformation and of transcription factors that act as sensors for toxicants or cellular damage[3].

The defence mechanism against natural and synthetic xenobiotics has long been investigated in arthropod species because of their great economic, environmental and medical-veterinary importance, as pollinators, agricultural pests, and vectors of human and animal diseases. Over the last decades, synergistic, enzymatic, genetic, and transcriptional studies have highlighted the involvement of metabolic detoxification in both resistance and defence to insecticides in a wide range

of taxa[10–12]. Over-expression of genes encoding for proteins of the biotransformation system has been observed in insecticide resistant strains as well as transcriptional induction of defensome members has been observed in susceptible strains exposed to insecticides. More recently, whole transcriptome analyses, which allow to observe the turning on and off of thousands of genes in response to toxic compounds, are showing a role for previously overlooked gene families, such as genes encoding for Heat Shock Proteins (HSPs) and Cuticular Proteins (CPs)[13–15].

Despite the increased focus on the components of the chemical defence, the sequence of the events that occur during toxic exposure remains poorly understood. Transcriptomic studies that have focused on single time-points showed that the exposure of individuals to toxicants induces up-regulation of several genes, while several others are down-regulated[16–21]. However, these are single snapshots of the defence response that do not allow us to know if and how the differentially expressed genes are modulated during toxicant exposure. On the other hand, some studies have investigated the expression profiles of subsets of genes at different time-points during individuals' exposure to toxicants[14,22–26]. For example, in the mosquito *Culex quinquefasciatus*, multiple P450 genes were found co-upregulated in resistant strains during a time course of 12, 24, 48 and 72 hours of permethrin exposure[24,25]. Likewise, in larvae of a susceptible strain of the mosquito *Anopheles stephensi* exposed to permethrin, it was analysed the expression pattern of six ABC transporter genes at seven time-points from 30 minutes to 48 hours after exposure. All of these genes were found differentially expressed compared to the untreated larvae at each time-point and showed a modulated transcriptional response across time, with the maximum up-regulation after six hours of exposure[22]. Similar patterns were also found for genes encoding for Cuticular Proteins in the mosquito *Culex pipiens pallens*[23]. These studies support the view that defensome genes likely operate continuously, turning off and on at different time-points. However, because these studies are based on a subset of defensome genes, just a partial picture of the whole defensome choreography has been so far provided.

In light of the above, we used RNA-Seq to massively detect differentially expressed genes in larvae of the main urban Asian malaria vector *An. stephensi* after exposure to permethrin, one of the most used synthetic insecticides. We exposed larvae of a susceptible strain of this mosquito (Liston) to the LD_{50} dose of permethrin and analysed the transcriptional response of the survived larvae (i.e. those that more efficiently defended them-selves) after six, 24 and 48 hours of insecticide exposure. By using a high-throughput technique, i.e. RNA-Seq, with measurements taken at several time-points, we were able to obtain a more thorough picture of the chemical defensome, which allowed us to *i*) assess how many and which defensome genes were differentially expressed after insecticide exposure; *ii*) describe the transcriptional response across time of defensome gene families.

2. Results

Permethrin exposure. Third-instar larvae were exposed to 0.137 mg/L of permethrin[22]. Larval mortality after six, 24, and 48 hours of permethrin exposure was 33%, 45%, and 76% respectively. In control tests larval mortality was 2% after six- (C-6 h control-test), 2% after 24- (C-24 h control-test), and 1% after 48-hours of exposure (C-48 h control-test).

Three pools of fifty living larvae were collected from both control- and test-trays after six and 24 hours of permethrin exposure and stored for molecular analyses. Two pools of fifty larvae were collected from the 48 hours test-tray, because of the high larval mortality observed at this time-point.

Ten living larvae were collected from both test- and control-trays and used to assess the larval health status after exposure to permetrhin by analysing diving and feeding activities. The Shapiro-Wilk test showed normal distribution of the data (all tests $P > 0.05$). Diving rates observed were significantly lower (t-tests $P < 0.05$) in larvae exposed to permethrin than in untreated larvae at all the measured time-points. On the contrary, no significant differences in diving rates were observed between all control samples, as well as between treated samples at all time-points (t-tests $P > 0.05$). Likewise, the time spent for feeding was lower in larvae exposed to permethrin than in control larvae at each time-point (t-tests $P < 0.05$), while no significant differences were detected between all control samples as well as between treated samples at six, 24 and 48 hours (all t-tests $P > 0.05$) (Supplementary Table S1).

Sequencing. A total of 17 cDNA libraries were constructed and sequenced: 3 libraries for the three pools of larvae exposed to permethrin for six hours (T1–6 h, T2-6 h and T3-6 h) and 3 for the control pools (C1-6 h, C2-6 h and C3-6 h); 3 libraries for the three pools of larvae exposed to permethrin for 24 hours (T1-24 h, T2-24 h and T3-24 h) and 3 for the control pools (C1-24 h, C2-24 h and C3-24 h); 2 libraries for the two pools of larvae exposed to permethrin for 48 hours (T1-48 h, T2-48 h) and 3 for the control pools (C1-48 h, C2-48 h and C3-48 h) (Supplementary Table S2). High quality paired-end short reads obtained ranged from 8,075,568 (T3-6h library) to 26,742,198 (T2-48 h library) with an average number of reads of 16,544,552 (Table 1; Supplementary Table S2).

Mapping and Annotation. The percentage of reads mapping to the reference genome of *Anopheles stephensi* (AsteI2.2, Indian strain) ranged from 83.79% (T2-6 h library) to 89.09% (C1-24 h library) with an average mapping rate of 86.3% (Table 1 and Supplementary Table S2).

A total of 9,388 genes were found expressed. Annotation by BlastP search of *An. stephensi* genes against a custom mosquito protein database resulted in 8,033 proteins recognized with a significant e-value ($P < 1e^{-5}$). Proteins annotated using a Gene Ontology (GO) approach were 7,599, with 1,981 unique GO terms (Table 1). Additionally, we characterized 6,748 proteins with EuKaryotic

Orthologous Groups (KOG)[27] and 4,792 with BlastKOALA (4,136 unique Kyoto Encyclopedia of Genes and Genomes orthologies) (KEGG)[28]. A total of 272 insecticide defence-related genes were found (Supplementary Table S3). Among them, 30 ABC transporter genes, belonging to all known ABC subfamilies but ABCH, were identified using a BlastP search and classified using a phylogenetic approach (5 ABCA, 4 ABCB, 8 ABCC, 2 ABCD, 1 ABCE, 3 ABCF and 7 ABCG) (Supplementary Fig. S1).

Description	Value
Average number of reads	16,544,552
Average mapping rate - %	86.30
Expressed genes	9,388
Annotated genes – BLAST p-value $< 1^{e-5}$	8,033
Annotated genes – GO	7,599
Total unique GO terms	1,981
Annotated genes KOG	6,748
Annotates genes BlastKoala	4,792
Total unique KO	4,136

Table 1. Summary of *Anopheles stephensi* cDNA sequencing, mapping and annotation. GO: gene onthology; KOG: EuKaryotic Orthologous Groups; KO: KEGG orthology.

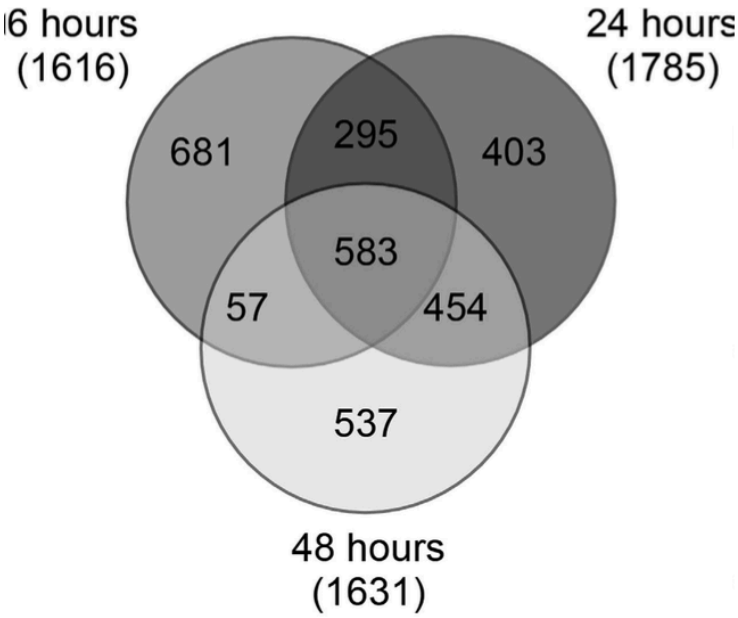


Figure 1. Venn diagram. Differentially expressed genes found in *Anopheles stephensi* in at least one time-point after permethrin exposure.

Differential Expression analysis. *Transcriptome changes during permethrin exposure*. Thirty-two per- cent of the 9,388 expressed genes (2,986/9,388) were differentially expressed (DE) in at least one time-point over the 48 hours of permethrin exposure. The Venn diagram in Fig. 1 shows the number of shared and exclusive DE genes at each time-point. About 20% (583 genes) of the DE

genes were expressed in response to permethrin over all of the 48 hours time-course, while 23% (681 genes) were differentially expressed exclusively at six hours after insecticide exposure, 13.5% (403 genes) were exclusive of 24 hours, and 18% (537 genes) exclusive of 48 hours after exposure. Some other genes were commonly expressed at six and 24 hours (9.8%), at 24 and 48 hours (15.2%), and at six and 48 hours (1.9%).

Functional transcriptome changes after permethrin exposure were assessed by classifying gene functions using a GO analysis. In total, forty-two GO terms of the three GO categories (cellular component, molecular function and biological process) were assigned across the 48 hours of exposure (Fig. 2). Among them, 35 sub-categories were assigned at six hours after permethrin exposure (9 in cellular component; 10 in molecular function, and 16 in biological process). The major sub-categories within each category were cell and cell part (324 GO terms), binding (618 GO terms) and metabolic process (513 GO terms), respectively. After 24 hours of insecticide expo- sure, 37 sub-categories were attributed as follows: 11 in cellular component (cell and cell part were the major sub-categories with 391 GO terms); 10 in molecular function (binding was the major sub-category with 614 GO terms); 16 in biological process (metabolic process was the major sub-category, 580 GO terms). After 48 hours of insecticide exposure, 40 sub-categories were found: 11 in cellular component (364 GO terms in cell and cell part); 10 in molecular function (587 GO terms in binding); 19 in biological process (518 GO terms in metabolic process) (Fig. 2).

In total, 2,048 out of the 2,986 differentially expressed genes were annotated into 25 KOG categories (Fig. 3). Among them, the cluster “general function prediction” was the largest (159; 28.26%, 163; 26.43%, 153; 27.15% at six, 24 and 48 hours of permethrin exposure, respectively), followed by “signal transduction mechanisms” (134; 24.44%, 130; 23.21%, 136; 25.31% at six, 24 and 48 hours, respectively) which also showed the highest number of up-regulated genes at all time points (Fig. 3). The smallest group was “cell motility” (1; 0.17%, 1; 0.14%, and 0 at six, 24 and 48 hours, respectively) followed by “nuclear structure” (8; 1.35%, 7; 1.07%, 6; 1.03%). Finally, 1,396 differentially expressed genes were characterized with 1,193 KEGG orthologies. Within the pathways belonging to “xenobiotics biodegradation and metabolism”, we found 52 differentially expressed genes. This subset included 22 defensome genes encoding for Phase 0/III, I and II enzymes, as well as 30 further genes which could be involved in the response to insecticide (Supplementary Table S3 and Table S4).

Defensome expression changes during permethrin exposure. A total of 272 genes involved in insecticide defence were found and among them 177 were differentially expressed in at least one time-point (Supplementary Table S3 and S4). Sixtysix Phase I genes were detected and among them 67% (44/66) were differentially expressed over the 48 hours of permethrin exposure (25, 22 and 30 genes were differentially expressed after six, 24 and 48 hours of exposure, respectively) (Table 2). Up- and down-regulated genes at the different time-points are shown in the Venn diagrams

in Supplementary Fig. S2. With respect to the up-regulated genes, three were found up- regulated at all time points analysed over the 48 hours time-course, while seven were up-regulated exclusively at six hours after insecticide exposure, one exclusively at 24 h and three exclusively at 48 hours (Supplementary Table S3 and Fig. S2). Thirtyfive Phase II genes were found and 68.5% (24/35) were differentially expressed in at least one time-point over the 48 hours of permethrin exposure (four, 20 and 23 genes after six, 24 and 48 hours of exposure, respectively) (Table 2).

Nineteen Phase 0/III genes encoding for ABC transporters, belonging to the ABCB, ABCC and ABCG sub- families, were found and 53% of them (10/19) were differentially expressed in at least one time-point (five, eight and seven genes after six, 24 and 48 hours, respectively) (Table 2). Two ABC transporter genes were up-regulated at all time-points analysed over the 48 hours time-course, while one gene was found up-regulated exclusively at six hours and one after 48 hours of exposure (Table 2; Supplementary Fig. S2).

In addition to Phase I, II and 0/III genes, other genes related to the defensome were found differentially expressed. Among these, genes enconding for antioxidant enzymes (one catalase and five superoxide dismutases encoding genes), transcription factors that act as sensors of toxicants or cellular damage, such as the aryl hydro- carbon receptor (Ahr), nuclear factors (NRs), the mitogen-activated protein kinase (MAPK) signaling pathways and the nuclear factor erythroid (Nfr2) have been detected (Supplementary Table S3). Finally, other xenobiotics defence-related genes and genes known to be involved in the general stress response were found differentially expressed, such as the genes encoding for Heat Shock Proteins (10 genes) and Cuticular Proteins (68 genes) (Table 2, Supplementary Table S3 and Fig. S2).

The temporal expression profiles of Phase I, II, and 0/III genes were further investigated by clustering the members of each sub-group based on their log fold-change (Fig. 4; Supplementary Fig. S3). Phase 0/III genes clustered in two main groups. The first one, made up of all up-regulated genes, was composed mostly of ABCG transporters (3 genes) and one ABCB transporter; in the second group, containing all down-regulated genes, only ABC transporters belonging to the ABCB and ABCC sub-families were found (Fig. 4A). The Phase I genes clustered into two main groups and several sub-groups containing both CYPs and CCEs genes. Some of them contained only up-regulated genes after at least one time-point, some others contained both up- and down-regulated genes or only down-regulated genes (Fig. 4B). All Phase II genes, with the exception of one UGT gene (ASTEI00013-RA) clustered into several sub-groups containing both GSTs and UGTs genes that were down-regulated mostly after 24 and 48 hours of exposure (Fig. 4C).

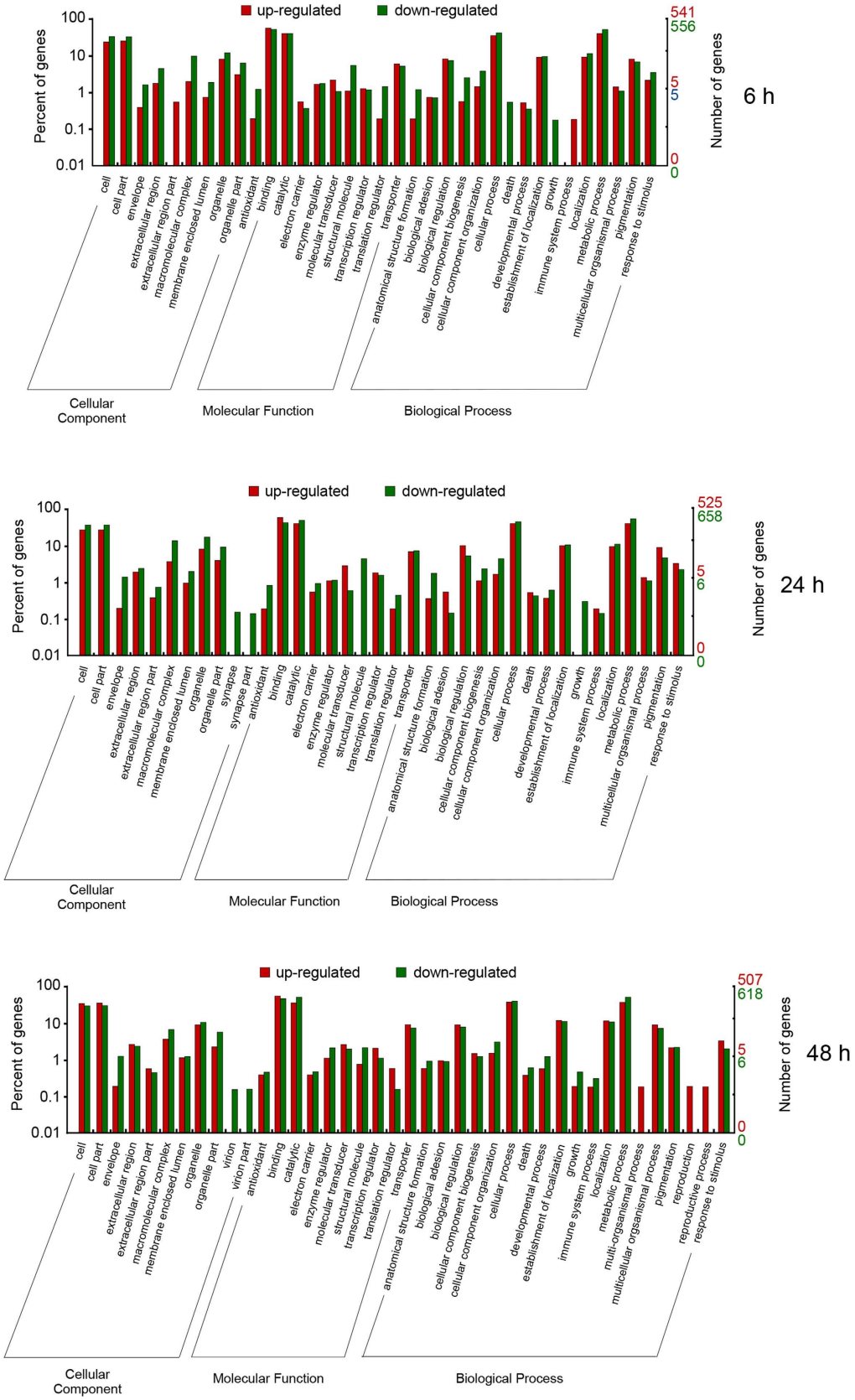


Figure 2. GO analysis by ontology categories. The fraction of genes classified in “Molecular function”, “Biological process” and “Cellular component” categories at six, 24 and 48 hours after permethrin exposure is shown.

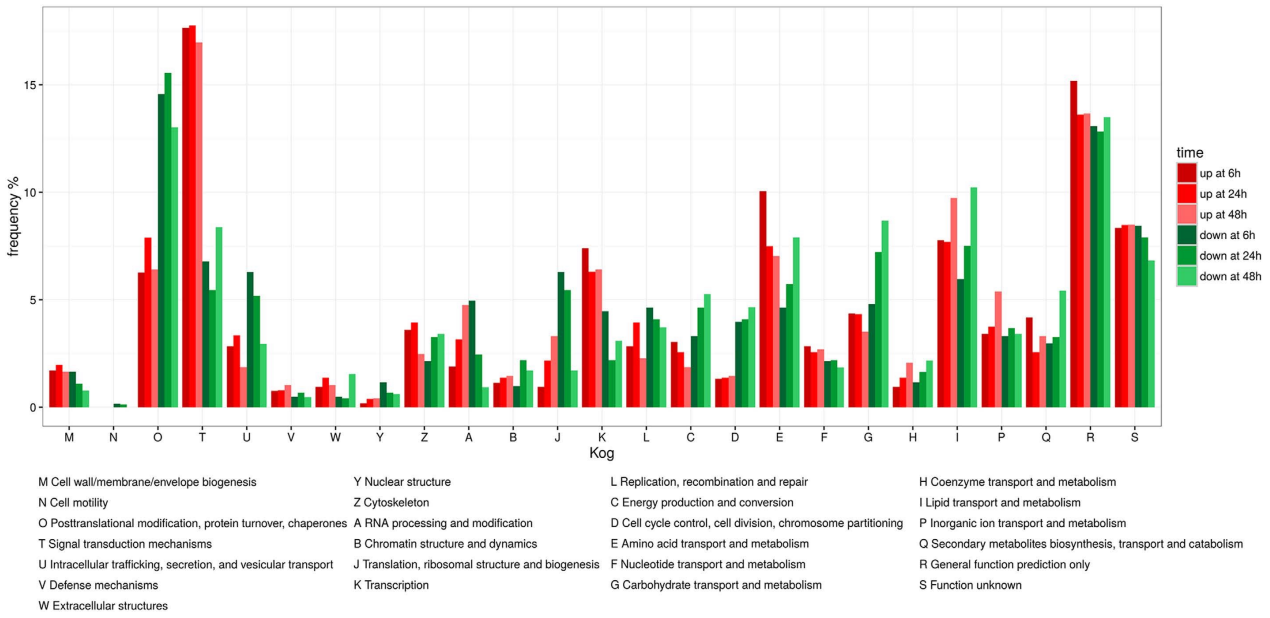


Figure 3. Clusters of Orthologous Groups function classification. Up- and down-regulated genes are shown for each functional category for six, 24 and 48 hours after permethrin exposure.

	N_{tot}	N_{DE}	N_{No-DE}	6h			24h			48h		
				Up	Down	No-DE	Up	Down	No-DE	Up	Down	No-DE
Phase I	**66**	**44**	**22**	**14**	**11**	**41**	**9**	**13**	**44**	**7**	**23**	**36**
Cytochromes P450 (CYPs)	44	33	11	9	9	26	6	9	29	5	18	21
Epoxide hydrolase (EHs)	1	0	1	0	0	1	0	0	1	0	0	1
Aldo-keto-reductase (AKRs)	1	0	1	0	0	1	0	0	1	0	0	1
Carboxylesterases (CCEs)	20	11	9	5	2	13	3	4	13	2	5	13
Phase II	**35**	**24**	**11**	**1**	**3**	**31**	**1**	**19**	**15**	**1**	**22**	**12**
Glutathione S-tansferases (GSTs)	22	14	8	0	2	20	0	13	9	0	14	8
UDP-glucuronosyltransferases (UGTs)	13	10	3	1	1	11	1	6	6	1	8	4
Phase 0/III	**19**	**10**	**9**	**3**	**2**	**14**	**2**	**6**	**11**	**3**	**4**	**12**
Antioxidant Enzymes	**6**	**2**	**4**	**1**	**0**	**5**	**1**	**1**	**4**	**1**	**1**	**4**
Catalases (CATs)	1	0	1	0	0	1	0	0	1	0	0	1
Superoxide dismutases (SODs)	5	2	3	1	0	4	1	1	3	1	1	3
Heat Shock Proteins (HPs)	**10**	**7**	**3**	**0**	**7**	**3**	**0**	**6**	**4**	**0**	**5**	**5**
Cuticular Proteins (CPs)	**68**	**54**	**14**	**5**	**33**	**30**	**0**	**3**	**65**	**5**	**11**	**52**

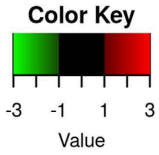
Table 2. Defensome genes in larvae of *Anopheles stephensi*. N_{tot}: total number of defensome genes found, genes differentially expressed (DE) in at least one time-point after permethrin exposure (N_{DE}) and no differentially expressed in any time-point (N_{NO-DE}). Up, Down and No-DE: genes up-, down-regulated and no differentially expressed with respect to controls after six, 24 and 48 hours of exposure to permethrin.

Cluster analysis was also performed for differentially expressed genes encoding for Cuticular Proteins (CPs), resulting in two main groups and several sub-groups. Cluster I contained all down-regulated genes but one, that was up-regulated at six hours of exposure. Cluster II was subdivided into further sub-groups. The IIa1 contained only genes up-regulated after 48 hours of exposure, while the IIa2 group contained only genes up-regulated after six hours. The group IIb contained only down-regulated genes after six hours of permethrin exposure (Supplementary Fig. S4).

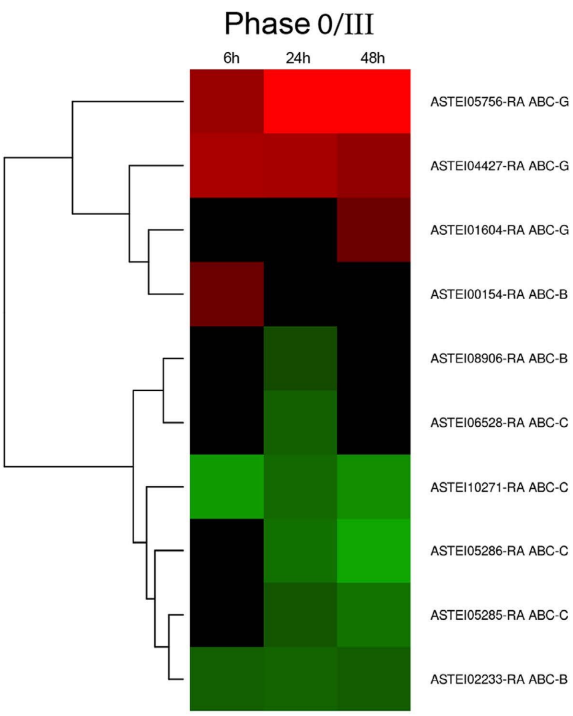
Reverse Transcription Quantitative Real-Time PCR (RT-qPCR) validation. Eight transcripts, detected as differentially expressed between treated and untreated mosquito larvae by RNA-Seq at different time-points were used for validation by RT-qPCR analysis. All genes showed a concordant pattern of up- or down-regulation between RNA-Seq and RT-qPCR data (Supplementary Fig. S5).

Discussion Defensome genes in *Anopheles stephensi* larvae. Permethrin, like all pyrethroid insecticides, is a neurotoxic compound whose main target is the voltage gated sodium channel[29]. In this study, we exposed larvae of a susceptible strain of the mosquito *Anopheles stephensi* to permethrin insecticide and analysed the transcriptional response after six, 24, and 48 hours of exposure to investigate the defensome response to insecticide stress across time. The involvement of detoxification complex enzymes in both resistance and defence to pyrethroids has been documented in several arthropod species, including mosquitoes[6,11,12]. Among them, cytochromes P450 (CYPs), carboxylesterases (CCEs), glutathione S-transferases (GSTs), UDP-glucuronosyl transferases (UGTs) enzymes, as well as ABC transporters were found differentially expressed between resistant and susceptible strains to pyrethroids[16–18,30,31]. Likewise, induced up-regulation of detoxifying genes was found in resistant individuals exposed to pyrethroids, such as in the mosquito *Anopheles gambiae*[30], the cattle tick *Rhipicephalus* (*Boophilus*) *microplus*[32] or the mosquito *Culex quinquefasciatus*[24,25], as well as in susceptible individuals, such as in *Panonychus citri*[19], *Liposcelis bostrychophila*[20], and *Melita plumulosa*[21].

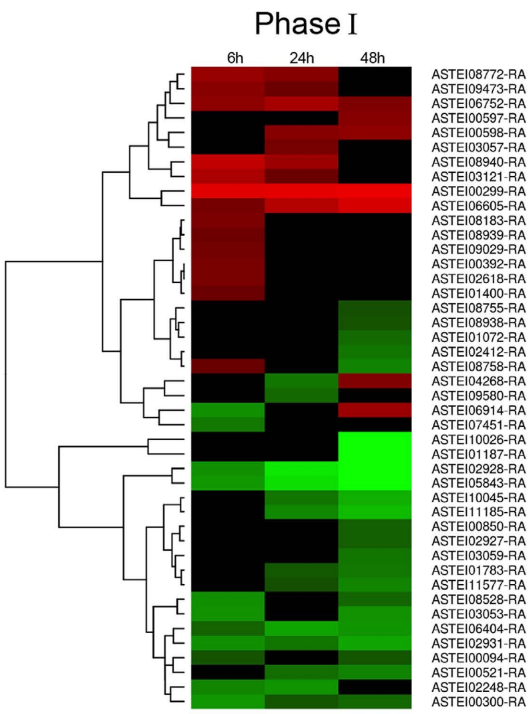
Concordantly with the above studies, we annotated 272 genes in the genome of *An. stephensi* involved in insecticide defence and found extensive transcriptional changes between larvae exposed and not-exposed to permethrin. Sixtysix Phase I genes were found, including several CYPs and CCEs, the main enzymatic complexes acting in this phase, and 29% of them were up-regulated in at least one time-point over the 48 hours of permethrin exposure, which supports a major role of these detoxifing enzymes in defence against permethrin. Phase II genes found included GSTs and UGTs (22 and 13 genes, respectively) and, among them, one UGT gene was up-regulated, while all GSTs were down-regulated in treated larvae. Down-regulation or no-differential expression of GSTs encoding genes after pyrethroid exposure has been documented in other insect species. For example, in third instar-nymphs of the rice planthoppers *Sogatella furcifera* exposed to the LD_{20}


Color Key
-3
-1
1
3
Value

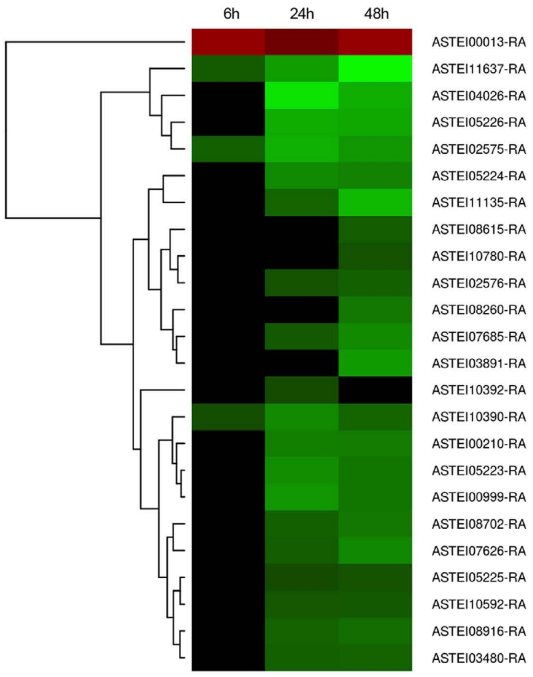
(A)


Phase 0/III
6h
24h
48h
ASTEI05756-RA ABC-G
ASTEI04427-RA ABC-G
ASTEI01604-RA ABC-G
ASTEI00154-RA ABC-B
ASTEI08906-RA ABC-B
ASTEI06528-RA ABC-C
ASTEI10271-RA ABC-C
ASTEI05286-RA ABC-C
ASTEI05285-RA ABC-C
ASTEI02233-RA ABC-B

(B)


Phase I
6h
24h
48h
ASTEI08772-RA
ASTEI09473-RA
ASTEI06752-RA
ASTEI00597-RA
ASTEI00598-RA
ASTEI03057-RA
ASTEI08940-RA
ASTEI03121-RA
ASTEI00299-RA
ASTEI06605-RA
ASTEI08183-RA
ASTEI08939-RA
ASTEI09029-RA
ASTEI00392-RA
ASTEI02618-RA
ASTEI01400-RA
ASTEI08755-RA
ASTEI08938-RA
ASTEI01072-RA
ASTEI02412-RA
ASTEI08758-RA
ASTEI04268-RA
ASTEI09580-RA
ASTEI06914-RA
ASTEI07451-RA
ASTEI10026-RA
ASTEI01187-RA
ASTEI02928-RA
ASTEI05843-RA
ASTEI10045-RA
ASTEI11185-RA
ASTEI00850-RA
ASTEI02927-RA
ASTEI03059-RA
ASTEI01783-RA
ASTEI11577-RA
ASTEI08528-RA
ASTEI03053-RA
ASTEI06404-RA
ASTEI02931-RA
ASTEI00094-RA
ASTEI00521-RA
ASTEI02248-RA
ASTEI00300-RA

(C)


Phase II
6h
24h
48h
ASTEI00013-RA
ASTEI11637-RA
ASTEI04026-RA
ASTEI05226-RA
ASTEI02575-RA
ASTEI05224-RA
ASTEI11135-RA
ASTEI08615-RA
ASTEI10780-RA
ASTEI02576-RA
ASTEI08260-RA
ASTEI07685-RA
ASTEI03891-RA
ASTEI10392-RA
ASTEI10390-RA
ASTEI00210-RA
ASTEI05223-RA
ASTEI00999-RA
ASTEI08702-RA
ASTEI07626-RA
ASTEI05225-RA
ASTEI10592-RA
ASTEI08916-RA
ASTEI03480-RA

(D)

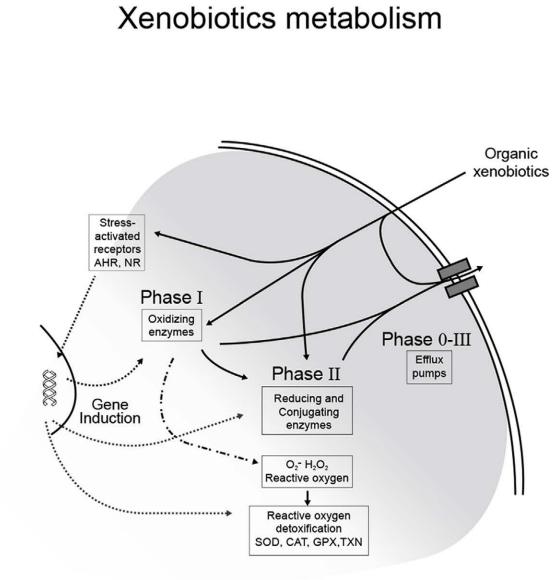

Xenobiotics metabolism
Organic xenobiotics
Stress-activated receptors AHR, NR
Phase I
Oxidizing enzymes
Phase 0-III
Efflux pumps
Phase II
Reducing and Conjugating enzymes
Gene Induction
O_2^- H_2O_2 Reactive oxygen
Reactive oxygen detoxification SOD, CAT, GPX,TXN

Figure 4. Differentially expressed genes in mosquito larvae exposed to permethrin at six, 24 and 48 h. Hierarchical clustering analysis based on their log fold-change. Genes not differentially expressed in any time-point were excluded from the analysis. For each gene, the ID is also indicated. (**A**) Phase 0/III enzymes: ABC transporters; (**B**) Phase I enzymes: Cytochrome P450 and Carboxylesterases; (**C**) Phase II enzymes: Glutathione S-transferases and UDP-glucuronosyltransferases. (**D**) Graphical representation of the chemical defensome. In the figure, the detoxifying metabolic enzymes and the pathways involved are shown. The figure was traced and modified from[1], using the software Canvas 15 (ACD systems http://www.acdsee.com/de/products/canvas-15).

---

concentration of beta-cypermethrin, three and six out of the nine GSTs genes found were detected no differentially expressed and down-regulated at 24 h, respectively[26]. Likewise, in the booklouse *Liposcelis bostrychophila* exposed to the LD_{20} dose of deltamethrin, none out of 31 GST genes found were differentially expressed between treated and untreated individuals[20]. GSTs have been suggested to catalyse different detoxification reactions, such as conjugation under exposure to organophosphates insecticides, or conjugation and dehydrochlorination in response to organochlorines[8]. Against pyrethroids, they would act by reducing peroxidative damage through detoxification of lipid peroxidation products. This indirect detoxifying role could account for their absent up-regulation in the time-window analysed in our study, concordantly with the scarce up-regulation observed in other genes encoding for antioxidant enzymes (one superoxide dismutase antioxidant gene) (Supplementary Table S3). In this context, it could be hypothesized that the constitutive activity of GSTs and antioxidant enzymes is enough to attenuate the accumulation of ROS generated by Phase I enzymes or that antioxidant transcriptional response could be activated in time-points out of the time window analysed under our experimental conditions[14]. Nineteen Phase 0/III genes were found, comprising members of all ABC-transporters sub-families known to be involved in insecticide detoxification[9], with four of them (21%) detected as up-regulated in at least one time-point of permethrin expo- sure. These results are concordant with our previous studies on induction of ABC transporters in *An. stephensi* by permethrin[22,33] and, more in general, with the even more numerous evidences for ABC transporters involvement in arthropod defence against insecticides[9,22,33–39].

It is interesting to note that some genes that we found differentially expressed in *An. stephensi* larvae had already been shown to be involved in pyrethroid resistance in mosquitoes, although caution should be taken when comparing different species, strains (resistant and susceptible) and results from different experimental designs (e.g. induced vs constitutive expression profile analyses). For example, the CYP352C2 gene (ASTEI00597-RA) that we found up-regulated, is known to be constitutively up-regulated in an *Anopheles arabiensis* strain resistant to deltamethrin[40]. On the other

hand, some other genes that we found down-regulated or not differentially expressed, were found constitutively up-regulated in a pyretroid-resistant strain of *An. stephensi*[41] (i.e. the GSTs ASTEI05223-RA, ASTEI09484-RA and ASTEI10780-RA; the CYP450, ASTEI02412-RA, and the CCEs, ASTEI08528-RA encoding genes) (Supplementary Table S3). The investigation of factors underlying the heterogeneity of defensome response between species or resistant and susceptible strains of the same species remains an unanswered and exciting topic for future researches[38].

Defensome genes include transcription factors that act as sensors of toxicants or cellular damage. We annotated and detected 38 genes encoding for transcription factors being expressed, including the aryl hydrocarbon receptor (Ahr), nuclear factors (NRs), mitogen-activated protein kinase (MAPK) and the nuclear factor erythroid (Nfr2). Among them, we detected up-regulation for one gene encoding for the Aryl hydrocarbon receptor interacting protein after 24 hours of exposure (ASTEI04432-RA) (Supplementary Table S3), a component of the Ahr pathways that regulate the Phase I genes expression, one gene encoding for a MAP kinase after 24 and 48 hours (ASTEI06997-RA), and one encoding for a Nrf2 after 24 hours (ASTEI05762-RA), which are both involved in the induction of genes encoding for antioxidant enzymes (Supplementary Tables S3)[3,42,43]. These results support the occurrence of all these signaling pathways in *An. stephensi* and their putative role in insecticide defence [see ref. 44]. Notably, among the DE genes, ten Cuticular Proteins were up-regulated in treated larvae, which further support the recent data showing their important role in insecticide defence and resistance[23,45,46].

Defensome transcriptional changes during permethrin exposure. Three major patterns can be observed in the transcriptional changes of defensome genes during permethrin exposure.

First, the greatest fraction of defensome genes differentially expressed after six, 24 and 48 hours of treatment was down-regulated (Table 2, Fig. 3, Supplementary Table S3). This pattern is particularly evident in Phase II genes, where all but one gene were down-regulated, but it can be also observed in Phase I and Phase 0/III genes, as well as in the genes encoding for HSPs (70% down-regulated), and CPs (65% down-regulated). Furthermore, this pattern can be observed at both all and single time-point analysed (Table 2). Permethrin exposure can be extremely costly from an energetic point of view. The target site of permethrin is the GABA receptor and the effect of its hyperactivation by permethrin leads to energy loss. Likewise, the defence response is energetically costly as a consequence of the transcriptional induction, protein synthesis, and detoxification activity and could lead to drastic metabolic changes in individuals exposed to insecticides and a reallocation of energy resources[47,48]. For example, in *Apis mellifera,* up-regulation of detoxifying genes by the neonicotinoid insecticide imidacloprid was coupled with down-regulation of genes associated with glycolisis and development[49]. Coherently with this, as suggested by the GO and KOG analyses, we found drastic

transcriptional changes in all categories and sub-categories throughout all times of exposure. For example, genes belonging to the GO growth sub-category were down-regulated after six and 24 hours (Fig. 2). Furthermore, we found a reduction of larval diving and feeding activities in exposed larvae at all time points compared to controls. These changes could therefore be part of a general reallocation of energetic resources which could account as well for the fraction of down-regulated defensome genes.

Secondly, when we look at the transcriptome changes during insecticide exposure, a modulated response of defensome genes across time can be observed at multiple levels. The first one was the gene level. The expression pattern of differentially expressed genes ranged from down- to up-regulation at all time-points, encompassing all possible combinations of up-/down-regulation at each time-point. For example, the ASTEI06914 gene (Phase I, CYP18A1) was down-regulated, not differentially expressed, and up-regulated at six, 24 and 48 hours, respectively. The opposite expression pattern was found in the ASTEI08758 gene (Phase I, CYP4H14). These results confirm and extend the findings of those studies that analysed across time the expression profile of genes involved in insecticide defence, such as the CYPs[24,25], the ABC transporters[22], or the CPs[23]. A further level of modulation of defensome response can be observed at the gene family level. Within each family, some genes were up- or down-regulated at all time-points, while others were differentially expressed only in one or two time-points (Fig. 3). Finally, a modulation of the transcriptional response from six to 48 hours of permethrin exposure can be observed at the whole defensome level. Groups of genes with similar expression patterns can be also observed when we consider all differentially expressed defensome genes. The gene groups and sub-groups found at each time-point include genes belonging to different gene families. Interestingly, it can also be observed that the response to insecticide is not due to high up-regulation of few genes, but rather to the up-regulation of several genes showing similar values of expression, suggesting the induction of multiple defensome members that overlap across time.

Finally, the number of up-regulated genes peaked at six hours and then decreased during exposure. At six hours of exposure 17% of defensome genes were up-regulated, while at 24 and 48 h of exposure the 10% and 12% of up-regulated genes were observed. The deleterious effects of permethrin as well as the energetic cost of detoxification could gradually weaken the larvae, reducing their defence response across time. At least three sets of evidences in our data led to consider this hypothesis unlikely: *i*) the results of larval activity tests showed no significant differences between the larvae exposed to permethrin for six, 24 and 48 hours, suggesting no differences in the health status of the surviving larvae between the time-points analysed (Supplementary Table S1); *ii*) the overall number of up-regulated genes decreased across time, but a coherent reduction according to their function can be observed between the detoxifying metabolic enzymes. For example, in Phase 0/III genes, slight differences were observed from six to 48 hours, which is consistent with the role

of the ABC transporters in defence response: on one hand they would act as the first line of cellular defence by pumping out from the cell the un-modified insecticide molecules; on the other hand, they act expelling the insecticides modified by Phase I and II enzymes at the end of the biotransformation process. Likewise, Phase I genes were mostly up-regulated after six hours of exposure and then their expression decreased at 24 and 48 hours, which is consistent with their role in insecticide metabolism (Table 2, Fig. 4D). *iii*) As discussed above, the transcriptional response is modulated at different levels: some genes and groups of genes turn off from six to 48 hours of treatment, but some others switch on across time, which can hardly be explained by a gradual larval weakening. On the contrary, these findings support the view that the observed temporal changes are due to a modulated defensome response across time, that would be stronger in the early stages of exposure and then be reduced with the ongoing of the detoxification process. It might be premature to speculate on the generality of the above patterns as the exposure conditions (i.e. time of exposure and toxicant dose) can greatly affect the defensome response, as well as it can differ among species, strains, developmental stages or sexes[14,38]. However, this study is the first attempt to give a picture of the temporal defensome expression dynamics during insecticide stress and provides a valuable resource for understanding how defensome genes work together.

Conclusions

Ecological transcriptomics has proved to be a powerful tool to investigate how organisms cope with xenobiotic stressors. Detoxification enzymes play important roles in the metabolism of insecticides in insects and they can rapidly increase their activity in response to chemical stress, a phenomenon known as enzyme induction. Transcriptional studies allowed to highlight that the induction of detoxification enzymes involves the synthesis of new enzymes rather than the activation of pre-existing enzymes or a block in the rate of degradation[4]. Indeed, genes encoding for proteins of the biotransformation system have been observed constitutively over-expressed in insecticide resistant strains while transcriptional induction of defensome members has been observed in susceptible strains exposed to insecticides[4,50]. More recently, whole transcriptome analyses greatly improved the detection of the genes involved in defence against insecticides. The analysis of changes in gene expression across time during chemical stress is the needed further step to move from gene inventories to the unraveling of gene interactions and regulation pathways. In this context, the results obtained in this study provide a first dynamic picture of defensome response to insecticides and a framework for future studies.

Materials and Methods

Mosquito samples and permethrin exposure. The mosquito larvae used in this study come from an *An. stephensi* (Liston) insecticide-susceptible strain maintained in the insectary of the University of Camerino, Italy. Newly hatched first instar larvae were maintained at 29 °C temperature, 85–90% relative humidity, 12:12 L-D photoperiod in 21 × 25 × 9 cm plastic trays filled with 2 liters of spring water and daily fed with fish food (Tetra, Melle, Germany)[33]. All experiments were conducted on *An. stephensi* third instar larvae.

In previous studies using the same *An. stephensi* strain, we analysed the transcriptional response of genes encoding for ABC transporters after 30 min., one, two, four, six, 24 and 48 hours of exposure to the LD_{50} dose of permethrin (0.137 mg/L). Significant gene up-regulation was detected mainly after six, 24 and 48 hours of exposure[22,33]. On the basis of these results, we treated the larvae with 0.137 mg/L of permethrin (PESTANAL®, $C_{21}H_{20}Cl_2O_3$, Sigma-Aldrich S.r.l., Milan, Italy) and analysed transcriptome changes by RNA-Seq after six, 24 and 48 hours of exposure.

Six experimental plastic trays (21 × 25 × 9 cm) filled with two liters of spring water and containing 500 larvae were set up: three trays for larval exposure tests (T-6 h, T-24 h, T-48 h, respectively) (0.137 mg/L permethrin), and three trays for the control tests without permethrin (C-6 h, C-24 h and C-48 h, respectively). After six hours of exposure, larval mortality was measured in test- and control-trays (T-6 h and C-6 h trays, respectively). Then, three pools of fifty living larvae were collected from the T-6 h test-tray (T1–6 h, T2-6 h and T3-6 h) and three from the C-6 h control-tray (C1-6 h, C2-6 h and C3-6 h), placed in RNAlater and stored at −80 °C for molecular analyses.

Ten additional larvae were collected from both test- and control-trays and used to assess larval health status after exposure to permetrhin. For this purpose, diving and feeding activities of larvae were analysed. Diving is a common larval behaviour that consists of bottom-up movements in the water column of breeding sites[51,52]. Single larvae were placed into 250 ml plastic glasses filled with 100 ml of the same water of the experimental tray (water + permethrin). After 1 minute of larval naturalization, we registered the number of divings within 5 minutes of observations. We defined feeding behaviour of larvae as three activities: movement to the food source, capture of food particles, and mouthpart movements. It was assessed by registering the time that each larva spent feeding within 5 minutes of each observation. Single larvae were placed into 250 ml plastic glasses filled with 100 ml of the same water of the experimental tray as above and containing 0.3 mg of fish food. The time spent feeding was then registered. Shapiro-Wilk test was performed to check normality of the data[53], then the Student's t-test was used for testing the differences between treated and control larvae and between the larvae exposed to permethrin at different time-points. All

analyses were performed using the software R 3.0.2[54].

The same procedures of permethrin exposure and activity assessment described above were followed using larvae exposed to permethrin for 24 (T-24 h tray) and 48 hours (T-48 h tray) and their respective controls (C-24 h and C-48 trays).

RNA isolation, cDNA library construction and Sequencing. RNA was extracted from each pool of larvae stored in RNAlater with the RNAeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. cDNA libraries preparation and sequencing were performed by Polo GGB, Perugia, Italy (http://www.pologgb.com/) in one run of 2 × 150 paired-end reads on a HiSeq-2500 platform (Illumina). Reads are available in the EBI Short Read Archive (Sample accession: ERS1203181- ERS1203197).

Reads mapping and Annotation. After an assessment of the reads quality using FastQC[55], high quality paired-end reads were mapped to the reference genome of *An. stephensi* retrieved from VectorBase (AsteI 2.2, Indian strain)[56] using Bowtie 2[57].

Gene sequences of the reference genome of *An. stephensi* were annotated using several approaches. Firstly, a similarity-based approach was used by creating a custom mosquito database of annotated proteins. Protein fasta files from five mosquito species (*Aedes aegypti, An. darlingi, An. gambiae, An. sinensis,* and *Culex quinquefasciatus*) were retrieved from VectorBase and unannotated sequences were removed. A BlastP[58] search of *An. stephensi* proteins against this database was performed with cutoff e-value of 1E-05. Secondly, GO terms and putative domains were assigned to *An. stephensi* sequences using InterproScan5[59].

Additionally, *An. stephensi* proteins were further characterized using the KOG and KEGG databases. Sequences were classified in one of the 25 KOG functional categories using Blast with a cutoff e-value of 1E-05. KEGG mapping was performed using the BlastKOALA webserver, while KEGG Mapper was used to reconstruct individual pathways. In addition, we exploited the already published annotation from the microarray study on *An. stephensi*[41] to validate or expand our defensome annotation. Genes encoding for detoxifying families were retrieved from the microarray chip and identified in our reference genome with a double best hit blastn search. Finally, for a more confident assignment of ABC transporters to ABC sub-families, we mined the genome of *An. stephensi* for putative transporters. A comprehensive custom dataset was created by retrieving all ABC transporter sequences in Uniprot using the taxonomic filter Metazoa. *An. stephensi* ABC transporters were identified with a BlastP search against this database. Successively, putative *An. stephensi* ABC transporters were classified into families with a phylogenetic approach by creating a multi-alignment of all ABC transporter sequences, putative and Uniprot annotated, with Cobalt[60] and using the result

as input for RaxML[61] with 1000 bootstrap replicates.

Differential Expression analysis. Raw counts for each gene and each sample were extracted from SAM alignments using samtools[62] and htseq-count[63]. A table of raw counts was used as input for DESeq2[64] for the normalization step and differential expression analysis. Samples were analyzed separately following the DESeq2 software documentation, as they are distinct pools of different individuals randomly sampled (quasi replicates). Pairwise comparisons were made between controls and treated samples at each time-point. At any given time-point, a gene was considered differentially expressed if its adjusted p-value (Benjamini-Hochberg adjustment) was less than 0.05 and its absolute log2 fold change was greater than 1. GO terms belonging to up- and down-regulated genes at each time-point were visualized using WeGO[65]. Similarly, the KOG characterization of differentially expressed genes was processed using R and visualized using ggplot2[66]. Finally, the expression profiles of defensome genes and Cuticular Proteins were investigated by clustering the members of each sub-group based on their log fold-change using gplots[67].

Reverse Transcription Quantitative Real-Time PCR (RT-qPCR) validation. The gene expression profile of eight differentially expressed genes obtained by RNA-Seq was validated by reverse transcription quantitative PCR (RT-qPCR). We chose at least one member of the detoxifying enzymatic families that we were interested in and that we observed being differentially expressed after insecticide exposure. Genes showing up- and/ or down-regulation have been selected to validate differential expression in both directions. cDNAs were synthesized starting from 200 ng of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen). The cDNA was used as template for RT-qPCRs using the primer pairs reported in Supplementary Table S4, derived from the sequences identified in the transcriptome. The amplification fragments obtained using standard PCR conditions and cycles were sequenced in order to confirm the specificity of the amplifications[68]. Quantitative RT-PCRs of target genes were performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with SYBR Green supermix (Bio-Rad), following the conditions reported in ref. 22.

References

1. Goldstone, J. *et al.* The chemical defensome: environmental sensing and response genes in the *Strongylocentrotus purpuratus* genome. *Dev Biol.* **300,** 366–84 (2006).
2. Goldstone, J. Environmental Sensing and Response Genes in Cnidaria: The Chemical Defensome in the Sea Anemone *Nematostella vectensis*. *Cell Biol. Toxicol*. **24,** 483–502 (2008).
3. Steinberg, C. E. W. Arms Race Between Plants and Animals: Biotransformation System in *Stress ecology: environmental stress as ecological driving force and key player in evolution* (ed. Steinberg, C. E. W.) *61–105* (Springer New York, 2012).
4. Feyereisen, R. Insect cytochrome P450 In *Comprehensive Molecular Insect Science Biochemistry and Molecular Biology* (ed. Gilbert, L. I.) Vol. 4, 1–77 (Oxford Elsevier, 2005).
5. Berenbaum, M. R. & Johnson, R. M. Xenobiotic detoxification pathways in honeybees. *Curr. Opin. Insect Sci.* **10,** 51–58 (2015).
6. Liu, N. Insecticide resistance in mosquitoes: impact, mechanisms and research directions. *Ann. Rev. Entomol*. **60,** 537–559 (2015).
7. Ranson, H. & Hemingway, J. Glutathione transferases In *Comprehensive Molecular Insect Science* (eds. Gilbert, L. I., Iatrou, K. & Gill, S. S.) 383–402 (Elsevier Oxford, 2005).
8. Che-Mendoza, A., Penilla, R. P. & Rodrìguez, D. A. Insecticide resistance and glutathione S-transferases in mosquitoes: A review. *Afr. J. Biotechnol.* **8,** 1386–1397 (2009).
9. Dermauw, W. & Van Leeuwen, T. The ABC gene family in arthropods: Comparative genomics and role in insecticide transport and resistance. *Insect Biochem. Mol. Biol.* **45,** 89–110 (2014).
10. Liu, N., Li, M., Gong, Y., Liu, F. & Li, T. Cytochrome P450s-their expression, regulation, and role in insecticide resistance. *Pestic. Biochem. Physiol.* **120,** 77–81 (2015).
11. Feyereisen, R., Dermauw, W. & Van Leeuwen, T. Genotype to phenotype, the molecular and physiological dimensions of resistance in arthropods. *Pest Biochem. Physiol*. **121,** 1–17 (2015).
12. Van Leeuwen, T. & Dermauw, W. The molecular evolution of xenobiotic metabolism and resistance in chelicerate mites. *Annu. Rev. Entomol*. **61,** 475–498 (2016).
13. Steinberg, C. E. W. Heat Shock Proteins: The Minimal, but Universal, Stress Proteome in *Stress ecology: environmental stress as ecological driving force and key player in evolution* (ed. Steinberg, C. E. W.) 107–127 (Springer New York, 2012).
14. Doganlar, O. & Doganlar, Z. B. Responses of antioxidant enzymes and heat shock proteins in *Drosophila* to treatment with a pesticide mixture. *Arch. Biol. Sci. Belgrade* **67,** 869–876 (2015).

15. Lv, Y. *et al.* Comparative transcriptome analyses of deltamethrin-susceptible and –resistant *Culex pipiens pallens* by RNA-seq. *Mol. Gen. Genomics* **291,** 309–21 (2015).
16. Mamidala, P. *et al.* RNA-Seq and molecular docking reveal multi-level pesticide resistance in the bed bug. *BMC Genomics* **13,** 6 (2012).
17. Abdalla, H. *et al.* Insecticide susceptibility and vector status of natural populations of *Anopheles arabiensis* from Sudan. *Trans. R. Soc. Trop. Med. Hyg*., **102,** 263–271 (2008).
18. Faucon, F. *et al.* Identifying genomic changes associated with insecticide resistance in the dengue mosquito *Aedes aegypti* by deep targeted sequencing. *Genome Res*. **25,** 1347–1359 (2015).
19. Niu, J. *et al.* Transcriptome analysis of the citrus red mite, *Panonychus citri*, and its gene expression by exposure to insecticide/acaricide. *Insect Mol. Biol*. **21,** 422–436 (2012).
20. Dou, W. *et al.* Mining genes involved in insecticide resistance of Liposcelis bostrychophila Badonnel by transcriptome and expression profile analysis. *PLoS ONE* **8,** e79878 (2013).
21. Hook, S. E., Osborn, H. L., Spadaro, D. A. & Simpson, S. L. Assessing mechanisms of toxicant response in the amphipod *Melita plumulosa* through transcriptomic profiling. *Aquat. Toxicol.* **146,** 247–57 (2014).
22. Epis, S. *et al.* Temporal dynamics of the ABC transporter response to insecticide treatment: insights from the malaria vector *Anopheles stephensi*. *Scientific Reports* **4,** 7435 (2014).
23. Fang, F. *et al.* The cuticle proteins: a putative role for deltamethrin resistance in *Culex pipiens pallens*. *Parasitol Res.* **114,** 4421–9 (2015).
24. Liu, N., Li, T., Reid, W. R., Yang, T. & Zhang, L. Multiple cytochrome P450 Genes: Their constitutive overexpression and permethrin induction in insecticide resistant mosquitoes, *Culex quinquefasciatus*. *PLoS ONE* **6,** e23403 (2011).
25. Gong, Y., Li, T., Gao, X. & Liu, N. Permethrin induction of multiple cytochrome P450 genes in insecticide resistant mosquitoes, *Culex quinquefasciatus*. *Int. J. Biol. Sci.* **9,** 863–871 (2013).
26. Zhou, W. W. *et al.* Genomic Insights into the glutathione S-transferase Gene Family of Two Rice Planthoppers, *Nilaparvata lugens* (Stål) and *Sogatella furcifera* (Horváth) (Hemiptera: Delphacidae). *PloS One*, **8,** e56604 (2013).
27. Tatusov, Roman L. *et al.* "The COG database: an updated version includes eukaryotes". *BMC bioinformatics 4.1*. 1 (2003).
28. Kanehisa, Minoru *et al.* "KEGG for integration and interpretation of large-scale molecular data sets". *Nucleic acids Res*. gkr988 (2011).
29. O'Reilly, A. O. *et al.* Modelling insecticide-binding sites in the voltage-gated sodium channel *Biochem. J.* **396,** 255–263 (2006).

30. Vontas, J. *et al.* Gene expression in insecticide resistant and susceptible *Anopheles gambiae* strains constitutively or after insecticide exposure. *Proc. Natl. Acad. Sci. USA*. **102,** 4080–4084 (2005).
31. David, J. P., Ismail, H. M., Chandor-Proust, A. & Paine, M. J. I. Role of cytochrome P450s in insecticide resistance: impact on the control of mosquito-borne diseases and use of insecticides on earth. *Phil. Trans. R. Soc. B* **368,** 20120429 (2013).
32. Saldivar, L. *et al.* Microarray analysis of acaricide-inducible gene expression in the southern cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*. *Insect Mol. Biol*. **17,** 597–606 (2008).
33. Epis, S. *et al.* ABC transporters are involved in defense against permethrin insecticide in the malaria vector *Anopheles stephensi*. *Parasit. Vectors* **7,** 349 (2014).
34. Buss, D.S. & Callaghan, A. Interaction of pesticides with p-glycoprotein andother ABC proteins: a survey of the possible importance to insecticide: herbicide and fungicide resistance. *Pestic. Biochem*. *Physiol.* **90,** 141–153 (2008).
35. Porretta, D. *et al.* Defence mechanism against insecticides temephos and diflubenzuron in the mosquito *Aedes caspius*: the P-glycoprotein efflux pumps. *Med. Vet. Entomol.* **22,** 48–54 (2008).
36. Pohl, P. C. *et al.* ABC transporter efflux pumps: a defence mechanism against ivermectin in *Rhipicephalus* (*Boophilus*) *microplus*. *Int. J. Parasitol.* **41,** 1323–1333 (2011).
37. Cafarchia, C. *et al.* Potential role of ATP-binding cassette transporters against acaricides in the brown dog tick *Rhipicephalus sanguineus* sensu lato. *Med. Vet. Entomol.* **29,** 88–93 (2015).
38. Porretta, D. *et al.* How heterogeneous is the involvement of ABC transporters against insecticides? *Acta Trop.* **157,** 131–5 (2016).
39. De Marco, L. *et al.* Transcriptome of larvae representing the *Rhipicephalus sanguineus* complex. *Mol Cell Probes*, doi: 10.1016/j. mcp.2016.02.006 (2016).
40. Muller, P. *et al.* Field-caught permethrin-resistant *Anopheles gambiae* overexpress CYP6P3, a P450 that metabolises pyrethroids. *PLoS Genet*. **4,** e1000286 (2008).
41. Vontas, J. *et al.* Transcriptional analysis of insecticide resistance in *Anopheles stephensi* using cross-species microarray hybridization. *Insect Mol. Biol.* **16,** 315–324 (2007).
42. Misra, J. R., Horner, M. A., Lam, G. & Thummel, C. S. Transcriptional regulation of xenobiotic detoxification in *Drosophila*. *Genes Dev*. **25,** 1796e1806 (2011).
43. Omiecinski, C. J., Heuvel, J. P. V., Perdew, G. H. & Peters, J. M. Xenobiotic metabolism, disposition, and regulation by receptors: From biochemical phenomenon to predictors of major toxicities. *Toxicol. Sci.* **120,** S49–S75 (2011).
44. Yadetie, F. *et al.* Chourrout conservation and divergence of chemical defense system in the

tunicate *Oikopleura dioica* revealed by genome wide response to two xenobiotics. *BMC Genomics* **2,** 55 (2012).

45. Nkya, T. E., Akhouayri, I., Kisinza, W. & David, J. P. Impact of environment on mosquito response to pyrethroid insecticides: facts, evidences and prospects. *Insect Biochem. Mol. Biol.* **43,** 407–416 (2013).
46. Vannini, L., Reed, T. W. & Willis, J. H. Temporal and spatial expression of cuticular proteins of *Anopheles gambiae* implicated in insecticide resistance or differentiation of M/S incipient species. *Parasit. Vectors* **7,** 24 (2014).
47. Zerba, E. Insecticidal activity of pyrethroids on insect of medical importance. *Parasitol Today* **4,** no.7 (1988).
48. Kliot, A. & Ghanim, M. Fitness costs associated with insecticide resistance. *Pest Manag. Sci*. **68,** 1431–7 (2012).
49. Derecka, K. *et al.* Transient exposure to low levels of insecticide affects metabolic networks of honeybee larvae. *PLoS One* **8,** e68191 (2013).
50. Yu, S. J. *The toxicology and biochemistry of insecticides*. (Boca Raton, FL: CRC Press 2008).
51. Clements, A. N. Behaviour and aspects of the biology of larvae In *The Biology of Mosquitoes: Sensory Reception and Behaviour*. 135–205 (Wallingford CABI Publishing, 1999).
52. Futami, K., Sonye, G., Akweywa, P., Kaneko, S. & Minakawa, N. Diving behavior in Anopheles gambiae (Diptera: Culicidae): avoidance of a predacious wolf spider (Araneae: Lycosidae) in relation to life stage and water depth. *J. Med. Entomol.* **45,** 1050–1056 (2008).
53. Patrick Royston Remark AS R94: A remark on Algorithm AS 181: The *W* test for normality. *Applied Statistics*, **44,** 547–551 (1995).
54. R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria 2013. http://www.R-project.org. Accessed 15 June 2016.
55. Andrews, S. FastQC: a quality control tool for high throughput sequence data. Available online at: http://www.bioinformatics. babraham.ac.uk/projects/fastqc (2010).
56. Jiang, X. *et al.* Genome analysis of a major urban malaria vector mosquito *Anopheles stephensi*. *Genome Biol*. **15,** 459 (2014).
57. Langmead, B. & Salzberg, S. L. Fast gapped-readalignment with Bowtie 2. *Nature Methods* **9,** 357–359 (2012).
58. Camacho, C. *et al.* BLAST+: architecture and applications. *BMC bioinformatics* **10,** 421 (2009).
59. Jones, P. *et al.* InterProScan 5: genome-scale protein function classification. *Bioinformatics* **30,** 1236–1240 (2014).

60. Papadopoulos, J. S. & Agarwala, R. COBALT: constraint-based alignment tool for multiple protein sequences. *Bioinformatics* **23,** 1073–1079 (2007).
61. Stamatakis, A. RAxMLversion 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30,** 1312–1313 (2014).
62. Li, H. *et al.* The sequence alignment/map format and SAMtools. *Bioinformatics* **25,** 2078–2079 (2009).
63. Anders, S., Pyl, P. T. & Huber, W. HTseq — a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31,** 166–169 (2015).
64. Love, M. I., Huber, W. & Simon, A. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biol*. **15,** 1–21 (2014).
65. Ye, J. *et al.* WEGO: a web tool for plotting GO annotations. *Nucleic Acids Res*. **34,** W293–297 (2006).
66. Wickham, Hadley. ggplot2: elegant graphics for data analysis. Springer Science & Business Media, 2009.
67. Warnes, G. R. *et al.* gplots: Various R programming tools for plotting data. *R package version* **2.4** (2009).
68. Bazzochi, C. *et al.* Molecular and serological evidences for the circulation of the tick symbiont *Midichloria* (Rickettsiales: Midichloriaceae) in different mammalian species. *Parasit. Vectors* **6,** 350 (2013).

PAPER 2:

Gene expression modulation of ABC transporter genes in response to permethrin in adults of the mosquito malaria vector *Anopheles stephensi*

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

2017; 171:37–43 DOI:10.1016/j.actatropica.2017.03.012

Gene expression modulation of ABC transporter genes in response to permethrin in adults of the mosquito malaria vector *Anopheles stephensi*

Valentina Mastrantonio[1,a], Marco Ferrari[2,a], Sara Epis[2,5,*], Agata Negri[1], Giulia Scuccimarra[1], Matteo Montagna[3], Guido Favia[4], Daniele Porretta[1], Sandra Urbanelli[1], Claudio Bandi[2,5]

[1]Department of Environmental Biology, Sapienza University of Rome, Via dei Sardi 70, 00185 Rome, Italy

[2]Department of Biosciences, University of Milan, Via Celoria 26, 20133 Milan, Italy

[3]Department of Agricultural and Environmental Sciences, University of Milan, Via Celoria 2, 20133 Milan, Italy

[4]School of Bioscience and Veterinary Medicine, University of Camerino, Via Gentile III da Varano, 62032 Camerino, Italy

[5]Pediatric Clinical Research Center Romeo and Enrica Invernizzi, Ospedale “Luigi Sacco”, Via Giovanni Battista Grassi, 74, 20157 Milan, Italy

*Corresponding author at: Department of Biosciences, University of Milan, Via Celoria 26, 20133 Milan, Italy. E-mail address: sara.epis@unimi.it (S. Epis).

[a]Co-primary authorship.

Received: 5 January 2017; Received in revised form: 10 March 2017; Accepted: 11 March 2017; Available online: 14 March 2017

Abstract

Living organisms have evolved an array of genes coding for detoxifying enzymes and efflux protein pumps, to cope with endogenous and xenobiotic toxic compounds. The study of the genes activated during toxic exposure is relevant to the area of arthropod vector control, since these genes are one of the targets upon which natural selection acts for the evolution of insecticide resistance. ATP-binding cassette (ABC) transporters participate to insecticide detoxification acting as efflux pumps, that reduce the intracellular concentration of toxic compounds, or of their metabolic derivatives. Here we analysed the modulation of the expression of six genes coding for ABC transporters, after the exposure of adult females and males of the mosquito *Anopheles stephensi*, a major malaria vector in Asia, to permethrin. Male and female mosquitoes were exposed to insecticide for one hour, then the expression profiles of the ABC transporter genes AnstABCB2, AnstABCB3, AnstABCB4, AnstABCBmember6, AnstABCC11, and AnstABCG4 were analysed after one and 24 h. Our results showed that three genes (AnstABCB2, AnstABCBmember6, AnstABCG4) were up-regulated in both sexes; two of these (AnstABCBmember6 and AnstABCG4) have previously been shown to be up-regulated also in larval stages of *An. stephensi*, supporting a role for these genes in permethrin defence in larvae as well as in adults. Finally, the same ABC transporter genes were activated both in females and males; however, the timing of gene induction was different, with a prompter induction in females than in males.

1. Introduction

Arthropods defend themselves from physiological metabolites and from both natural (e.g. plant and microbial toxins) and anthropic exogenous toxicants (e.g. organic pollutants and pesticides) by detoxifying protein systems, encoded by an array of genes and gene families; these systems, composed by detoxifying enzymes and efflux pumps, transform the toxic chemicals and/or eliminate them from cells (Goldstone, 2008; Steinberg, 2012). Identifying which genes and gene-family members are activated during the detoxification response is relevant to the control of arthropod vectors, as genes coding for detoxifying proteins are one of the substrates upon which natural selection acts for the evolution of insecticide resistance. In addition, detoxifying enzymes and transporters have the potential to be exploited as targets for the development of novel insecticides (Buss and Callaghan, 2008; Le Goff et al., 2006; Steinberg, 2012).
In recent years, ATP-binding cassette (ABC) transporters, integral membrane proteins belonging to the ABC family, have attracted a great deal of attention (Dermauw and Van Leeuwen, 2014; Merzendorfer, 2014). Differently from detoxifying enzymes (e.g. cytochrome-P450, or glutathione-S transferases), ABC transporters participate to cell defence not by modifying toxic substrates, but reducing their intracellular concentration, by extruding toxicants out of the cells once they have entered into them, or after that they have been modified by detoxifying enzymes (DermauwandVan Leeuwen, 2014; Merzendorfer, 2014). Among the ABC transporter families, particularly important are the ABC transporters belonging to the ABCB (also called MDRs or P-gps), ABCC and ABCG subfamilies (Dermauw and Van Leeuwen, 2014) that are considered the “first line of cellular defence” against a wide range of xenobiotics, including insecticides (Buss and Callaghan, 2008).

As already emphasized, the interest about ABC transporters in arthropods derives on one hand from the need to understand the molecular basis of insecticide defence, on the other hand from the perspective to discover new molecular targets for novel insecticides. In addition, these transporters could be exploited to improve the efficacy of ‘conventional’ insecticides, thanks to the synergistic effects with ABC transporters inhibitors (Buss and Callaghan, 2008; Cafarchia et al., 2015; Dermauw and Van Leeuwen, 2014; Pohl et al., 2011; Porretta et al., 2016). Indeed, sequence-specific gene silencing by RNA interference (RNAi), in different blood-sucking insect species, has shown the possibility to specifically suppress the ABC transporter gene expression, increasing the susceptibility of tested individuals (Dalla Bona et al., 2016; Figueira-Mansur et al., 2013; Yoon et al., 2011; Zhu et al., 2013).

In mosquitoes, ABC transporters have been shown to be involved in defence against several insecticide classes (Dermauw and Van Leeuwen, 2014). Bioassays using insecticides and ABC transporter inhibitors (e.g. verapamil or cyclosporin A) have shown that insecticide toxicity increased after the inhibition of these transporters. For example, increased larval mortality was observed in

larvae of susceptible populations of the mosquitoes *Aedes caspius* and *Aedes aegypti* exposed to temephos or in *Culex pipiens* larvae exposed to endosulfan, ivermectin and cypermethrin (Buss et al., 2002; Figueira-Mansur et al., 2013; Lima et al., 2014; Porretta et al., 2008). Likewise, in both Anopheles and Aedes species, up-regulation of ABC transporter genes has been observed in mosquito larvae from susceptible populations after insecticide exposure, supporting the view that ABC transporters play an important role in mosquito defence against chemicals (Epis et al., 2014a, b; Figueira-Mansur et al., 2013). For example, in *Ae. aegypti* larvae exposed to temephos, the authors observed a significant increase of the expression of the ABCB transporter AaegP-gp, up to eight-fold after 48 h (Figueira-Mansur et al., 2013).

Anopheles stephensi is the main malaria vector in the Middle East and South Asian regions (Sinka et al., 2010; WHO, 2016a). In this species, ABC transporters have been implicated in the defence against the pyrethroid insecticide permethrin, in larvae belonging to an insecticide-susceptible strain (Epis et al., 2014a). Furthermore, the analysis of the transcriptional response of six ABC transporter genes, identified in the larval transcriptome, showed both up- and down-regulation at different time points, which suggests the occurrence of a modulated response of ABC transporter genes during permethrin exposure.

To date, despite pyrethroids occupy a prominent position in adult control, studies focused on ABC transporters and pyrethroid insecticides in An. stephensi adults are limited (Vontas et al., 2007). Mosquito larvae and adults are naturally exposed to different endogeneous and xenobiotic compounds (Clements, 2000). The modulation of ABC transporter gene expression, in response to toxicants exposure, can thus be expected to differ in different life stages of the mosquito, as observed in other insect species. For example, the overexpression of an ABCG gene was observed in adults of the whitefly *Bemisia tabaci*, while no overexpression was observed in eggs and nymphae (Yang et al., 2013), which agrees with the idea that ABC transporter response to insecticides varies during insect development. On the other hand, in the salmon louse *Caligus rogercresseyi* the ABC transporter gene Cr-Pgp was found up-regulated in females after delthametrin exposure, but not in males, suggesting that differential responses are possible not only relative to the developmental stage, but also to sex (Valenzuela-Mun˜ozetal., 2014).

The aim of this work was to investigate the transcriptional response of ABC transporter genes to permethrin in adults of *An. stephensi*. Male and female mosquitoes were exposed to permethrin separately; then the expression profiles of six ABC transporter genes were determined, at two time-points after insecticide expo- sure (one and 24 h). Gene expression data were analysed to assess the possible occurrence of differences across time and/or between sexes, and discussed in relation with the results obtained on larvae of *An. stephensi* in previous studies (Epis et al., 2014a, b).

2. Material and methods

2.1 Mosquitoes

All mosquitoes used in this study derived from the *Anopheles stephensi* Liston colony of the insectary of the University of Camerino, Italy. Mosquitoes were reared at 28±1°C with a relative humidity of 85–90%, and a 12:12 Light-Dark photoperiod. All experiments were conducted separately on adult males and females. At this aim, the eggs were kept in spring water until hatching; larvae were fed daily with fish food (Tetra, Melle, Germany) and sex-separated as pupae; groups of 25 male and female adults were maintained in different cages and fed with a 5% sucrose solution until laboratory bioassays.

2.2 Mosquitoes exposure to permethrin

In order to analyse the expression profile of ABC transporter genes after permethrin exposure, male and female adults were treated with permethrin following the WHO tube protocol (WHO, 2016b) with some modifications. All experiments were carried out separately for male and female mosquitoes using 3–4 day-old unfed individuals, maintained at the same temperature, humidity and photoperiod conditions of the reared colony. Permethrin (PESTANAL, Sigma-Aldrich S.r.l., Milan, Italy) was diluted in acetone to have a final concentration of 0.45% that, accordingly to WHO (2016b) and our preliminary treatments, leads to about 50–80% mortality rate after 24 h. Twenty-five males or females were gently placed into separate tubes and exposed for one hour to papers (12 × 15 cm) (Whatman® No. 1, Sigma-Aldrich S.r.l., Milan, Italy) impregnated with 2ml of permethrin solution mixed with the silicon oil non-volatile carrier (Sigma-Aldrich S.r.l., Milan, Italy), accordingly to WHO (2016b). Papers impregnated with the silicone oil carrier and acetone (i.e. without insecticide) were used as controls. Seven replicates were conducted for permethrin treatments and four for controls.

ABC transporter gene expression was analysed at two time-points (one and 24 h). In a first experiment we exposed adult males and females to permethrin for one hour as described above, then the number of dead mosquitoes was recorded in both the exposure and control tubes. A mosquito was considered dead if it was immobile or unable to stand or fly in a coordinated way. The survived individuals in both the exposure and the control tubes were collected, divided into three pools of 10 individuals for controls and three for exposed individuals and stored in RNA later at −80°C for RNA extraction and molecular analysis. In the second experiment, we exposed adult males and females to permethrin for one hour. Then, the survived individuals were transferred in recovery tubes and supplied with sucrose solution. After 24 h, the number of dead mosquitoes in the recovery tubes was recorded and the survived individuals were collected for treatment and control (three pools

Gene	Forward primer (3'-5')	Reverse primer (3'-5')	Size (base pairs)	Source
AnstABCB2	TATCAAGTTCACGGATGTAGAGT	TATCCACCTTGCCACTGTC	185	Epis et al. (2014a)
AnstABCB3	CAACCGTTCCGTAATACTACC	ACTGGTAGCCCAATGTGAAG	133	Epis et al. (2014a)
AnstABCB4	GGACAAAACATTCGGGAGG	CGTAGTGAATGTTGTGGCG	109	Epis et al. (2014a)
AnstABCBmember6	CTGGAGACGCTGAGAGATA	TACTCCTCGGTGAACTGG	125	Epis et al. (2014a)
AnstABCG4	ATGAGCCCATTCGTCCTG	AGCGTGGAGAAGAAGCAG	158	Epis et al. (2014a)
AnstABCC11	GGTTGGATTGGCTTTCGTG	ATAACCGACTCCCGTTTCG	156	Epis et al. (2014b)
Rps7	AGCAGCAGCAGCACTTGATTTG	TAAACGGCTTTCTGCGTCACCC	90	Capone et al. (2013)

Table 1 Primer sequences used to amplify ABC transporter genes in *Anopheles stephensi*.

of 10 individuals for treatment and three for control) and stored in RNA later at −80 ° C until molecular analysis. Shapiro-Wilk test was performed to check the normality of the data, then the Student's t-test was used to assess the occurrence of significant differences in mortality rates between females and males at the two time-points after permethrin exposure. All tests were performed using the software IBM SPSS Statistics (IBM Corp. Released, 2013).

2.3 Gene expression profiles after permethrin exposure

The expression profiles of six genes encoding for ABC transporters (AnstABCB2, AnstABCB3, AnstABCB4, AnstABCBmember6, AnstABCC11, AnstABCG4) were analysed by Quantitative RT-PCRs in adult males and females at two time-points after permethrin exposure (one and 24 h). These genes have been identified in the larval transcriptome of *An. stephensi* and were found differentially expressed in larvae exposed to the LD_{50} dose of permethrin by Epis et al. (2014a, b). RNA was extracted from each pool of treated and untreated males and females using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Then the total RNA was eluted into nuclease-free water and the concentration of RNA was determined at 260 nm using Qubit^{TM} fluorometric quantitation (Thermo Scientific, Delaware, USA). cDNAs were synthesized from 150ng of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) with random hexamers. The cDNA was used as template in Quantitative RT- PCRs using the primers designed on *An. stephensi* by Epis et al. (2014a, b) (Table 1). Quantitative RT-PCRs on target genes were performed using a BioRad CFX Real-Time PCR Detection System (Bio-Rad, California, USA) and the conditions were as follows: 50 ng cDNA; 300nM of forward and reverse primers; 98°C for 30s, 40 cycles of 98°C for 15s, 59°C for 30s; fluorescence acquisition at the end of each cycle; melting curve analysis after the last cycle. In order to calculate the expression of the target genes, cycle threshold (Ct) values were determined for each gene and normalized according to the endogenous reference gene rps7. The expression of ABC transporters genes in the controls was considered as the basal level (equal to 1). The estimates of the expression level of each gene in treated males and females are relative to the control groups, and are reported as fold change mean ± standard deviation (SD). Significant differences in expression between treated and controls were

estimated using a two-sided t-test, as implemented in the CFX Manager software (Bio-Rad). The normality of the expression data was tested by Shapiro-Wilk test (Royston, 1995), then the univariate ANOVA was performed to compare differences in relative expression between sexes and time points for each gene that was found differentially expressed in both males and females after insecticide exposure. Subsequent pairwise comparisons were performed using the Student t-test. For multiple tests, the significance threshold (5%) was corrected by applying the Bonferroni correction. All analyses were performed using the software IBM SPSS Statistics (IBM Corp. Released, 2013).

Table 2 Univariate ANOVA analysis on relative gene expression in relation to sex and time.

Gene	*df*	MS	F	*P*
ABCG4				
Sex	1	3.876	285.527	0.000***
Time	1	8.841	651.258	0.000***
Sex × Time	1	0.087	6.387	0.035*
ABCB2				
Sex	1	25.172	33.164	0.000***
Time	1	30.528	40.221	0.000***
Sex × Time	1	20.593	27.131	0.001***
ABCBmember6				
Sex	1	15.413	14.608	0.005**
Time	1	6.660	6.312	0.036*
Sex × Time	1	44.545	42.216	0.000***

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

3. Results

The results of the bioassays revealed a higher sensitivity to permethrin of adult males of *An. stephensi*, compared to females. Indeed, mortality in males was 70% (±11%) and 82% (±7%), respectively at one and at 24 h after permethrin exposure, while mortality in females was 40% (±8%) and 55% (±12) at the two time-points. In the control tests, a mortality rate of 2% (±1) and 3% (±2) was found at one and 24 h, respectively. The Shapiro-Wilk test supported a normal distribution of the data (all tests P > 0.05) and the Student's t-test showed significant differences in mortality rate between males and females at one and 24 h (Student's t-tests P < 0.05) (Table S1).

The expression profiles of the analysed ABC transporter genes in adult males and females, at one and 24 h, are shown in Fig. 1 and in Table S2. The AnstABCB3, AnstABCB4 and AnstABCC11 genes were not differentially expressed or down-regulated compared to controls in both sexes after permethrin exposure. On the contrary, the AnstABCG4, AnstABCB2 and AnstABCBmember6 genes were up- regulated in both females and males at one and 24 h (Fig. 1).

The Shapiro-Wilk test showed normal distribution of the data (all tests P>0.05). The ANOVA analysis performed on the AnstABCG4, AnstABCB2 and AnstABCBmember6 genes showed that gene expression level was affected by time, sex and the combination of these two factors (Table 2).

Pairwise comparisons between females and males showed significant differences in the expression of AnstABCG4, AnstABCB2 and AnstABCBmember6 genes in the two sexes at one hour (Student t-tests P = 0.002, P = 0.02 and P = 0.002, respectively), while no differences were observed at 24 h (Fig. 1).

In treated females, the relative expression of the AnstABCG4, AnstABCB2 and AnstABCBmember6 genes at one hour were increased 4.93 ± 0.08, 8.51 ± 1.17 and 8.75 ± 1.69 fold, respectively (mean ± standard deviation), with AnstABCG4 showing the lowest increase in expression compared to control. At 24 h, the three genes showed an up-regulation of 3.38±0.18 (AnstABCG4), 9.15±0.15 (AnstABCB2) and 6.45±0.15 (AnstABCBmember6) fold (Fig. 1).

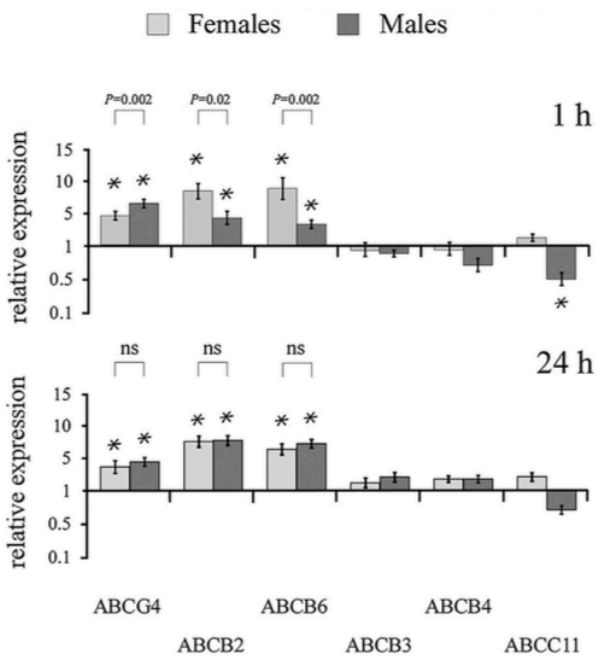


Fig. 1 Relative expression of *Anopheles stephensi* ABC transporter genes in female and male adults measured by Quantitative RT-PCR. The expression level in non-treated individuals was considered to be the basal level (equal 1). The internal reference gene rps7 for *An. stephensi* was used to normalize the expression levels. The values are expressed as fold-change compared to control and reported as mean of three values plus standard deviation. Light grey: females; dark grey: males. ABCG4: AnstABCG4; ABCB2: AnstABCB2; ABCB6: AnstABCBmember6; ABCB3: AnstABCB3; ABCB4: AnstABCB4; ABCC11: AnstABCC11 (Table 1). Asterisks show significant differences in over-expression between treated and control groups (P-value <0.05). Letters refer to comparison of the expression levels observed at one and 24 h for each gene in females and males. P-values of Student-t-tests between expression levels of females and males at one and 24 h are also shown. NS = no significant differences.

The relative expression value of the AnstABCG4 gene significantly decreased from one to 24 h (Student t-test $P < 0.0002$), while no significant differences were observed between the expression values of the AnstABCB2 and AnstABCBmember6 genes at the two time-points (Student t-tests $P > 0.05$).

In males, at one hour, the relative expression of the AnstABCG4, AnstABCB2 and AnstABCBmember6 genes was 6.27 ± 0.09, 3.2 ± 0.17 and 2.7 ± 0.08 fold, respectively, with the AnstABCG4 showing the highest expression value. At 24 h, the relative expression values of the three genes were 4.4 ± 0.08, 8.99 ± 0.15 and 7.98 ± 0.09 fold (Fig. 1). The relative expression value of the AnstABCG4 gene significantly decreased from one to 24 h (Student t-test $P < 0.0001$), while the expression values of the AnstABCB2 and AnstABCBmember6 genes significantly increased (Student t-tests $P < 0.0001$).

4. Discussion

In our previous works, by analysing the expression profile of ABC transporter genes in larvae of *An. stephensi* exposed to permethrin, we showed that two genes (AnstABCG4 and AnstABCBmember6) were up-regulated after insecticide exposure, supporting a role of ABC transporters in defence against this pyrethroid insecticide. Furthermore, by analysing the expression profiles at different time-points after permethrin exposure, we showed the occurrence of a modulated transcriptional response, where single genes were up- or down-regulated at different time-points after permethrin exposure, as well as multiple genes were co-regulated at each time-point (Epis et al., 2014a, b). In this study, we focused on adults of *An. stephensi* and the results obtained integrated and extended the results obtained on the larvae.

Here, we exposed adult females and males to permethrin for one hour, and analysed the expression profiles of the same set of genes analysed in larval stages, at two time-points after exposure (one and 24 h). Three out of the six genes analysed (AnstABCG4, AnstABCB2 and AnstABCBmember6) were up-regulated in both sexes after one and 24 h, while the remaining three (AnstABCB3, AnstABCB4 and AnstABCC11) were not differentially expressed or down-regulated after insecticide treatment (Fig. 1). These results (i.e. only some genes among those analysed exhibit up-regulation) are concordant to what has been recently described about ABC transporters and, more in general, in genes involved in the detoxification process (e.g. Cytochrome P450, Cuticular proteins) (Epis et al., 2014b; Fang et al., 2015; Gong et al., 2013; Liu et al., 2011). Because of the detoxification process is energetically costly, this pattern could be due to a general reallocation of the energetic resources, as recently suggested by some authors (De Marco et al., 2017).

The up-regulation of the AnstABCG4, AnstABCB2 and AnstABCBmember6, here observed after permethrin exposure, supports the involvement of ABC transporters in defence against this

insecticide not only in larval stages of *An. stephensi*, but also in adults. Notably, we found not only evidences of permethrin-induction of ABC transporters genes in both larvae and adults, but also we observed that five out of the six genes analysed showed similar expression profiles between larvae and adults after permethrin exposure: the AnstABCB3, AnstABCB4 and AnstABCC11 genes were indeed not differentially expressed or down-regulated after permethrin exposure in both life-stages, while the AnstABCG4 and AnstABCBmember6 genes were up-regulated in both adults and larvae (Fig. 1) (Epis et al., 2014a,b). The AnstABCB2 gene was found up-regulated in adults at both one and 24 h (Fig. 1), while this gene was not differentially expressed or down-regulated in *An. stephensi* larvae (Epis et al., 2014a, b). Although different experimental conditions (e.g. insecticide concentration or time of insecticide exposure) can affect gene expression induction, the results reported in Epis et al. (2014a, b) and in this study show that part of the ABC transporter genes investigated display a similar expression pattern after permethrin exposure in adult mosquitoes and larvae, while other genes are characterized by a stage-specific response.

The discovery that mosquito larvae and adults share ABC transporter genes that display a similar induction response after insecticide exposure is relevant to insect vector and pest control programmes, for at least two reasons. First, pyrethroids occupy a prominent position in malaria vector control as they are used to control adult mosquitoes on long-lasting insecticidal nets (LLINs) and indoor residual spraying programmes (IRS) (WHO, 2016a). However, pyrethroids are also widely used in crop pest control, so that also larval stages of anopheline mosquitoes can be exposed to pyrethroids in the developing sites near crop fields, which could increase the risk of insecticide resistance development (Bigoga et al., 2012; Gnankiné et al., 2013; Yadouleton et al., 2011). Therefore, the presence of genes displaying the same induction pattern in larval and adult stage would add a further critical factor for insecticide resistance insurgence, as these 'co- regulated' genes offer a common target for mutational events that can led to resistance development (e.g. point mutations, duplications, mutations in promoter/regulatory regions) (Le Goff et al., 2006; Liu, 2015; Nkya et al., 2013). Second, the inhibition of ABC transporter gene expression by RNAi technologies has shown the possibility to increase the susceptibility of individuals to insecticides by specifically targeting detoxifying genes (Figueira-Mansur et al., 2013; Yoon et al., 2011; Zhu et al., 2013). In perspective, this approach could allow to reduce the dose and frequency of insecticide applications (Buss and Callaghan, 2008; Porretta et al., 2008). It is interesting to note that dsRNA-based gene silencing in mosquito larvae can determine a long-lasting down-regulation of target genes, up to the adult stage (e.g. Dalla Bona et al., 2016). The occurrence of genes displaying the same induction pattern in larvae and adults, after insecticide treatment, thus adds further appeal to control strategies based on sequence-specific suppression of ABC transporter gene expression, as well as highlights the need to assess which genes are induced in the different life-stages to implement such control strategies.

When we look at the expression profiles of ABC transporters in female and male adults some similarities can be observed: in both sexes, among the six genes analysed, the AnstABCG4, AnstABCB2 and AnstABCBmember6 genes were up-regulated at both one and 24h after permethrin exposure. Furthermore, in both sexes the relative expression of the AnstABCG4 significantly decreased from one to 24h. Finally, at 24h, females and males showed a similar pattern of gene expression, where the genes AnstABCB2 and AnstABCBmember6 were the most up-regulated. However, differences can also be observed between the two sexes, in particular in terms of the temporal dynamics of the gene induction. In females, the relative expression of the AnstABCG4, AnstABCB2 and AnstABCBmember6 genes was similar at one and 24 h and no significant differences were found between the relative expression values of the AnstABCB2 and AnstABCBmember6 genes at the two time-points. In males, on the contrary, the AnstABCG4, AnstABCB2 and AnstABCBmember6 genes showed a modulated up-regulation across time and significant differences were observed in the relative expression of each gene at the two time-points: the induction of the AnstABCB2 and AnstABCBmember6 genes significantly increased from one to 24 h after exposure, while the opposite was observed in the AnstABCG4 gene (Fig. 1). Females, therefore, seem to have a prompter induction response than males and show high gene up-regulation since one hour after exposure (Fig. 1). Notably, we also found that females had a higher percentage of survival than males after insecticide treatment at both one and 24 h (Table S1), which could suggest an important role of the temporal dynamics of ABC transporter gene expression in the efficiency of defence response. Future studies, aimed to investigate the full set of ABC transporter genes recently identified in *An. stephensi* (De Marco et al., 2017), could allow to further investigate the occurrence of temporal shift in gene expression between females and males and the possible factors underlying it. Interestingly, ABC transporters have been shown to mediate both heme and pesticide detoxification in the tick *Rhipicephalus microplus* (Lara et al., 2015). We might hypothesize that the need for females to detoxify the heme released during blood digestion determines a condition of pre-adaptation in terms of the expression of proteins involved in detoxification, making them more resistant to insecticides. It would thus be interesting to analyse the expression profiles of ABC transporter genes in *An. stephensi* during and after the blood meal.

5. Conclusions

This study presents novel results on the expression of ABC transporter genes in response to insecticide treatment in the malaria vector *An. stephensi*, throughout experiments on adult mosquitoes of the two sexes. Three genes were shown to be induced in both females and males after permethrin exposure, among which two of them have previously been shown to be up-regulated also in larvae of the same *An. stephensi* Liston colony. Furthermore, the analysis of the expression profiles at two time-points allowed us to detect sex-based differences in gene regulation, with a prompter gene induction in female mosquitoes.

References

Bigoga, J.D., Ndangoh, D.N., Awono-Ambene, P.H., Patchoke, S., Fondjo, E., Leke, R.G.F., 2012. Pyrethroid resistance in Anopheles gambiae from the rubber cultivated area of Niete, South Region of Cameroon. Acta Trop. 124, 210–214, http://dx.doi.org/10.1016/j.actatropica.2012.08.010.

Buss, D.S., Callaghan, A., 2008. Interaction of pesticides with p-glycoprotein and other ABC proteins: a survey of the possible importance to insecticide, herbicide and fungicide resistance. Pestic. Biochem. Physiol. 90, 141–153.

Buss, D.S., McCaffery, A.R., Callaghan, A., 2002. Evidence for p-glycoprotein modification of insecticide toxicity in mosquitoes of the Culex pipiens complex. Med. Vet. Entomol. 16, 218–222, http://dx.doi.org/10.1046/j.1365-2915.2002. 00365.x.

Cafarchia, C., Porretta, D., Mastrantonio, V., Epis, S., Sassera, D., Iatta, R., Immediato, D., Ramos, R.A.N., Lia, R.P., Dantas-Torres, F., Kramer, L., Urbanelli, S., Otranto, D., 2015. Potential role of ATP-binding cassette transporters against acaricides in the brown dog tick Rhipicephalus sanguineus sensu lato. Med. Vet. Entomol. 29, 88–93, http://dx.doi.org/10.1111/mve.12093.

Capone, A., Ricci, I., Damiani, C., Mosca, M., Rossi, P., Scuppa, P., Crotti, E., Epis, S., Angeletti, M., Valzano, M., Sacchi, L., Bandi, C., Daffonchio, D., Mandrioli, M., Favia, G., 2013. Interactions between Asaia, Plasmodium and Anopheles: new insights into mosquito symbiosis and implications in malaria symbiotic control. Parasites Vectors 6, 182, http://dx.doi.org/10.1186/1756-3305-6-182.

Clements, A.N., 2000. The biology of mosquitoes, volume 2: Sensory reception and behaviour. In: Transactions of the Royal Society of Tropical Medicine and Hygiene., http://dx.doi.org/10.1016/s0035-9203(00)90448-6, p. 82.

Dalla Bona, A.C., Faitta Chitolina, R., Lopes Fermino, M., de Castro Poncio, L., Weiss, A., Perreira Lima, J.B., Paldi, N., Soares Bernardes, E., Henen, J., Maori, E., 2016. Larval application of sodium channel homologous dsRNA restores pyrethroid insecticide susceptibility in a resistant adult mosquito population. Parasites Vectors 9, 397, http://dx.doi.org/10.1186/s13071-016-1634-y.

De Marco, L., Sassera, D., Epis, S., Mastrantonio, V., Ferrari, M., Ricci, I., Comandatore, F., Bandi, C., Porretta, D., Urbanelli, S., 2017. The choreography of the chemical defensome response to insecticide stress: insights into the *Anopheles stephensi* transcriptome using RNA-Seq. Sci. Rep. 7, 41312–41323, http://dx.doi.org/10.1038/srep41312.

Dermauw, W., Van Leeuwen, T., 2014. The ABC gene family in arthropods: comparative genomics and role in insecticide transport and resistance. Insect Biochem. Mol. Biol. 45, 89–110.

Epis, S., Porretta, D., Mastrantonio, V., Comandatore, F., Sassera, D., Rossi, P., Cafarchia, C., Otranto, D., Favia, G., Genchi, C., Bandi, C., Urbanelli, S., 2014a. ABC transporters are involved in defense against permethrin insecticide in the malaria vector *Anopheles stephensi*. Parasites Vectors 7, 349, http://dx.doi.org/ 10.1186/1756-3305-7-349.

Epis, S., Porretta, D., Mastrantonio, V., Urbanelli, S., Sassera, D., De Marco, L., Mereghetti, V., Montagna, M., Ricci, I., Favia, G., Bandi, C., 2014b. Temporal dynamics of the ABC transporter response to insecticide treatment: insights from the malaria vector *Anopheles stephensi*. Sci. Rep. 4, 7435–7439, http://dx. doi.org/10.1038/srep07435.

Fang, F., Wang, W., Zhang, D., Lv, Y., Zhou, D., 2015. The cuticle proteins: a putative role for deltamethrin resistance in *Culex pipiens pallens*. Parasitol. Res. 114, 4421–4429, http://dx.doi.org/10.1007/s00436-015-4683-9.

Figueira-Mansur, J., Ferreira-Pereira, A., Mansur, J.F., Franco, T.A., Alvarenga, E.S.L., Sorgine, M.H.F., Neves, B.C., Melo, A.C.A., Leal, W.S., Masuda, H., Moreira, M.F., 2013. Silencing of P-glycoprotein increases mortality in temephos-treated *Aedes aegypti* larvae. Insect Mol. Biol. 22, 648–658, http://dx.doi.org/10.1111/ imb.12052.

Gnankiné, O., Bassolé, I.H.N., Chandre, F., Glitho, I., Akogbeto, M., Dabiré, R.K., Martin, T., 2013. Insecticide resistance in *Bemisia tabaci* Gennadius (Homoptera: Aleyrodidae) and *Anopheles gambiae* Giles (Diptera: Culicidae) could compromise the sustainability of malaria vector control strategies in West Africa. Acta Trop. 128, 7–17.

Goldstone, J.V., 2008. Environmental sensing and response genes in cnidaria: the chemical defensome in the sea anemone *Nematostella vectensis*. Cell Biol. Toxicol. 24, 483–502, http://dx.doi.org/10.1007/s10565-008-9107-5.

Gong, Y., Li, T., Gao, X., Liu, N., 2013. Permethrin induction of multiple cytochrome P450 genes in insecticide resistant mosquitoes, *Culex quinquefasciatus*. Int. J. Biol. Sci. 9, 863–871, http://dx.doi.org/10.7150/ijbs.6744.

IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.

Lara, F.A., Pohl, P.C., Gandara, A.C., Ferreira, J.D.S., Nascimento-Silva, M.C., Bechara, G.H., Sorgine, M.H.F., Almeida, I.C., Vaz, I.D.S., Oliveira, P.L., 2015. ATP binding cassette transporter mediates both heme and pesticide detoxification in tick midgut cells. PLoS One 10 (8), e0134779.

Le Goff, G., Hilliou, F., Siegfried, B.D., Boundy, S., Wajnberg, E., Sofer, L., Audant, P., ffrench-Constant, R.H., Feyereisen, R., 2006. Xenobiotic response in *Drosophila melanogaster*: sex dependence of P450 and GST gene induction. Insect Biochem. Mol. Biol. 36, 674–682,

http://dx.doi.org/10.1016/j.ibmb.2006.05.009.

Lima, E.P., Goulart, M.O.F., Rolim-Neto, M.L., 2014. Evaluation of the role of ATP-binding cassette transporter as a defence mechanism against temephos in populations of *Aedes aegypti*. Mem. Inst. Oswaldo Cruz 109, 964–966, http://dx. doi.org/10.1590/0074-0276140164.

Liu, N., Li, T., Reid, W.R., Yang, T., Zhang, L., 2011. Multiple cytochrome P450 genes: their constitutive overexpression and permethrin induction in insecticide resistant mosquitoes, *Culex quinquefasciatus*. PLoS One 6 (6), e23403, http://dx. doi.org/10.1371/journal.pone.0023403.

Liu, N., 2015. Insecticide resistance in mosquitoes: impact, mechanisms, and research directions. Annu. Rev. Entomol. 60, 537–559, http://dx.doi.org/10. 1146/annurev-ento-010814-020828.

Merzendorfer, H., 2014. ABC Transporters and their role in protecting insects from pesticides and their metabolites. In: Cohen, E. (Ed.), Advances in Insect Physiology. Target Receptors in the Control of the Insect Pests: Part II. Academic Press, London, pp. 1–73.

Nkya, T.E., Akhouayri, I., Kisinza, W., David, J.P., 2013. Impact of environment on mosquito response to pyrethroid insecticides: facts, evidences and prospects. Insect Biochem. Mol. Biol. 43, 407–416, http://dx.doi.org/10.1016/j.ibmb.2012. 10.006.

Pohl, P.C., Klafke, G.M., Carvalho, D.D., Martins, J.R., Daffre, S., da Silva Vaz, I., Masuda, A., 2011. ABC transporter efflux pumps: a defense mechanism against ivermectin in *Rhipicephalus (Boophilus) microplus*. Vet. Immunol. Immunopathol. 41, 1323–1333, http://dx.doi.org/10.1016/j.ijpara.2011.08.004.

Porretta, D., Gargani, M., Bellini, R., Medici, A., Punelli, F., Urbanelli, S., 2008. Defence mechanism against insecticides temephos and diflubenzuron in the mosquito *Aedes caspius*: the P-glycoprotein efflux pumps. Med. Vet. Entomol. 22, 48–54, http://dx.doi.org/10.1111/j.1365-2915.2008.00712.x.

Porretta, D., Epis, S., Mastrantonio, V., Ferrari, M., Bellini, R., Favia, G., Urbanelli, S., 2016. How heterogeneous is the involvement of ABC transporters against insecticides? Acta Trop. 157, 131–135, http://dx.doi.org/10.1016/j.actatropica. 2016.02.002.

Royston, P., 1995. Remark AS R94: a remark on algorithm AS 181: the W test for normality. App. Stat. 44, 547–551, http://dx.doi.org/10.2307/2986146.

Sinka, M.E., Bangs, M.J., Manguin, S., Coetzee, M., Mbogo, C.M., Hemingway, J., Patil, A.P., Temperley, W.H., Gething, P.W., Kabaria, C.W., Okara, R.M., Van Boeckel, T., Godfray, H.C.J., Harbach, R.E., Hay, S.I., 2010. The dominant Anopheles vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic précis. Parasites Vectors 3,

117, http:// dx.doi.org/10.1186/1756-3305-3-117.

Steinberg, C.E.W., 2012. Arms race between plants and animals: biotransformation system. In: Steinberg, C.E.W. (Ed.), Stress Ecology: Environmental Stress as Ecological Driving Force and Key Player in Evolution. Springer, New York, pp. 61–105.

Valenzuela-Munoz, V., Nunez-Acuna, G., Gallardo-Escárate, C., 2014. Molecular characterization and transcription analysis of P-glycoprotein gene from the salmon louse *Caligus rogercresseyi*. J. Aquac. Res. Dev. 5, 236–242, http://dx.doi. org/10.4172/2155-9546.1000236.

Vontas, J., David, J.P., Nikou, D., Hemingway, J., Christophides, G.K., Louis, C., Ranson, H., 2007. Transcriptional analysis of insecticide resistance in *Anopheles stephensi* using cross-species microarray hybridization. Insect Mol. Biol. 16, 315–324, http://dx.doi.org/10.1111/j.1365-2583.2007.00728.x.

WHO, 2016a. Eliminating Malaria. WHO Press, Geneva, Switzerland. WHO, 2016b. Test Procedures for Insecticide Resistance Monitoring in Malaria Vector Mosquitoes, 2nd ed. WHO Press, Geneva, Switzerland.

Yadouleton, A., Martin, T., Padonou, G., Chandre, F., Asidi, A., Djogbenou, L., Dabiré, R., Aïkpon, R., Boko, M., Glitho, I., Akogbeto, M., 2011. Cotton pest management practices and the selection of pyrethroid resistance in *Anopheles gambiae* population in northern Benin. Parasites Vectors 4, 60, http://dx.doi.org/10. 1186/1756-3305-4-60.

Yang, N., Xie, W., Jones, C.M., Bass, C., Jiao, X., Yang, X., Liu, B., Li, R., Zhang, Y., 2013. Transcriptome profiling of the whitefly *Bemisia tabaci* reveals stage-specific gene expression signatures for thiamethoxam resistance. Insect Mol. Biol. 22, 485–496, http://dx.doi.org/10.1111/imb.12038.

Yoon, K.S., Strycharz, J.P., Baek, J.H., Sun, W., Kim, J.H., Kang, J.S., Pittendrigh, B.R., Lee, S.H., Clark, J.M., 2011. Brief exposures of human body lice to sublethal amounts of ivermectin over-transcribes detoxification genes involved in tolerance. Insect Mol. Biol. 20, 687–699, http://dx.doi.org/10.1111/j.1365- 2583.2011.01097.x.

Zhu, F., Gujar, H., Gordon, J.R., Haynes, K.F., Potter, M.F., Palli, S.R., 2013. Bed bugs evolved unique adaptive strategy to resist pyrethroid insecticides. Sci. Rep. 3, 1456, http://dx.doi.org/10.1038/srep01456.

PAPER 3:

ABCG4 silencing in *Anopheles stephensi* larvae increases insecticide effectiveness

Manuscript in preparation for submission

ABCG4 silencing in *Anopheles stephensi* larvae increases insecticide effectiveness

Ferrari M. et al.

ABSTRACT

ABC transporters demonstrated to be involved in the detoxification of several compounds including permethrin, the most used pyrethroid for mosquito bed-nets treatment, indoor residual spraying (IRS) and for crop spraying. In particular ABCG4 gene, a member of the G family, showed to be always up-regulated in response to insecticide treatment in *Anopheles stephensi* individuals, both adults and larvae. To verify the importance of this transmembrane transporter in the detoxification process of permethrin, the gene has been down-regulated through specie-specific SiRNA (RNA interference (RNAi). The down-regulation of the gene led to an increased mortality in larvae, confirming a major role of ABCG4 transporter in the detoxification process against the selected insecticide. Moreover, this study underlines the presence of a systemic RNAi effect in mosquito larvae and the persistence through the developmental stages to adulthood, although the mechanism of diffusion remains unknown. In conclusion, targeting ABCG4 gene for silencing through RNAi technique is a promising strategy for increasing pyrethroids effectiveness in vector control.

INTRODUCTION

Vector-borne diseases are one of the main public health threats in the world. According to the WHO, 216 million cases of malaria and 445000 deaths occurred in 2016 (WHO 2017). Although great results have been achieved in the past decades with a reduction of the number of deaths by 29% between 2010 and 2015, the insurgence of artemisinin resistance in *Plasmodium falciparum* (Anderson et al., 2010; Fairhurst et al., 2016; Tilley et al., 2016) and insecticide resistance in mosquito populations (Alonso & Tanner, 2013; Karunamoorthi, 2011; Tikar et al., 2011) are threatening the efforts for an effective control of the disease and a malaria free world. Although the presence of resistant vector mosquito populations due to the heavy use of insecticides, these chemicals remain the core of all malaria control programs. Pyrethroids and permethrin in particular are widely used for Internal Residual Spraying (IRS) and for the treatment of bed-nets. For this reason, it is of pivotal importance to understand the mechanisms at the base of detoxification in mosquitoes, both sensitive and resistant, to develop strategies aimed to restore sensitivity in resistant populations and to avoid the development of resistance in sensitive ones.

Many studies have identified several genes involved in the detoxification of xenobiotics in mosquitoes, such as Glutathione-S-transferase (David et al., 2005; Tikar et al., 2009; De Marco et al., 2017), CYPs (Weill et al., 2004; De Marco et al., 2017), Epsilon glutathione transferase (Rooker et al., 1996), cuticular genes (Lumjuan et al., 2011) and ATP Binding Cassette (ABC) transporters (Epis et al., 2014a; Epis et al., 2014b; Mastrantonio et al., 2017). For some of these genes a down-regulation through RNA interference (RNAi) has been achieved inducing an increase of mosquito sensitivity to different classes of insecticides (Zhang et al., 2010; Figueira-Mansur et al., 2013; Singh et al., 2013), but this technique has also been used for identifying new resistance candidate genes (Ingham et al., 2014) tested as a biopesticide (Kumar et al., 2013) and as sterility inducer (Whyard et al., 2015), highlighting the potential of RNAi as a promising strategy in vector control.

In this study, RNAi has been applied to determine the role of the gene ABCG4 in larvae and adults of *An. stephensi.* This transporter, belonging to the G family of ABCs, is always up-regulated in response to permethrin treatment indicating an important role in detoxification against this insecticide in *An. stephensi* larvae (Epis et al., 2014a; Epis et al., 2014b; De Marco et al., 2017) and adults (Mastrantonio et al., 2017).

MATHERIAL AND METHODS

Mosquitoes breading. Eggs derived from a colony of susceptible mosquitoes belonging to the species *An. stephensi*, Linston strain, were obtained from the insectarium of the University of Camerino, Italy. In this colony adult mosquitoes are reared with a 12:12 Light-Dark photoperiod, following standard condition of temperature and humidity: 28 ± 1°C and 85-90% relative humidity, 5% sucrose solution feeding for adults and mouse blood for adult female before oviposition. Eggs were put into well water for hatching and larvae were fed daily with fish food (Tetra, Melle, Germany) following the same standard conditions maintained for adults.

Specific siRNA design. 25nt siRNA sequence (5' UCUACACACUGUACUGGCUCAUGUA 3') was designed using the online software BLOCK-IT^{TM} RNAi Designer (Thermo Fisher Scientific), with high complementarity to the ABCG4 mRNA sequence of *An. stephensi* (accession number LK392617.1). A scrambled sequence of the siRNA without homology to any *An. stephensi* gene has been used as control. Treatments with siRNA through oral delivery were performed on the third instar larvae according to WHO standard protocols (WHO, 2005).

Induction and silencing of ABCG4 in larvae. Groups of 50 third instar larvae were soaked in a volume of 357 µl siRNA or scrambled siRNA, without homology to any *An. stephensi* gene, at two different concentrations (0.03µg/µl and 0.06µg/µl) in RNase-free water to prevent siRNA degradation. Additional groups of 50 larvae were treated only with RNase-free water as control. This step was performed for three hours, and fish food (TetraFish) was administered to all groups. At the end of the three hours treatment, each group of larvae was gently transferred in 100 ml of well water and a LD_{50} dose of permethrin (0.072mg/l) has been added to all groups, except of two control groups. Before the administration, the powdered insecticide was previously dissolved in acetone and then diluted in water to obtain the test solutions for use.

Gene expression analysis in larvae. At 6 hours and 24 hours, pools of 5 surviving individuals (able to move through the water column) were put in extraction buffer+β-mercaptoethanol for immediate RNA extraction using RNeasy Mini Kit (Qiagen, Hilden, Germany) with an additional on-column DNase I treatment (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Extracted RNA concentration was determined by Qubit 3.0 Fluorometer (Thermo Fisher Scientific). cDNA was synthesized starting from 200ng of total RNA, using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) with random hexamers. cDNA was used as template in RT-qPCR reaction, with primers designed on ABCG4 gene sequence, already reported in previous works (Epis et al., 2014a, b). Two endogenous reference genes for *An. sthephensi* were used to obtain a normalization of data: Rps7 (Epis et al., 2014a) and GAPDH (Yamamoto et al., 2016) (Tab.1).

Gene target relative expression was detected using a BioRad iQ5 Real-Time PCR Detection System (Bio-Rad, California, USA). The analysis was carried out in accordance with the following conditions: 50 ng cDNA; 300 nM of forward and reverse primers; 98°C for 30'', 40 cycles of 98°C for 15'', 59°C for 30'', 72°C for 30''; fluorescence acquisition at the end of each cycle; melting curve analysis after the last cycle. Ct values were determined for each gene, in order to calculate gene expression levels of target gene using Rps7 and GAPDH as internal reference genes. The expression level of ABCG4 in the control group was considered as basal level (equal to 1), in order to evaluate the effect of permethrin induction and RNAi effect. The estimates of the expression level of ABCG4 in the siRNA-treated and scramble-treated larvae are reported as average between different pools ± standard deviation.

Table 1 Primer sequences of ABC transporters and housekeeping genes of *Anopheles stephensi*.

Gene	Forward primer	Reverse primer	bp	Source
*Anst*ABCB2	TATCAAGTTCACGGATGTAGAGT	TATCCACCTTGCCACTGTC	185	[Epis et al., 2014a]
*Anst*ABCB3	CAACCGTTCCGTAATACTACC	ACTGGTAGCCCAATGTGAAG	133	[Epis et al., 2014a]
*Anst*ABCB4	GGACAAAACATTCGGGAGG	CGTAGTGAATGTTGTGGCG	109	[Epis et al., 2014a]
*Anst*ABCBmember6	CTGGAGACGCTGAGAGATA	TACTCCTCGGTGAACTGG	125	[Epis et al., 2014a]
*Anst*ABCC11	GGTTGGATTGGCTTTCGTG	ATAACCGACTCCCGTTTCG	156	[Epis et al., 2014b]
*Anst*ABCG4	ATGAGCCCATTCGTCCTG	AGCGTGGAGAAGAAGCAG	158	[Epis et al., 2014a]
RPS7	AGCAGCAGCAGCACTTGATTTG	TAAACGGCTTTCTGCGTCACCC	90	[Capone et al., 2013]
GAPDH	GCCGTCGGCAAGGTCATCCC	TTCATCGGTCCGTTGGCGGC	166	[Yamamoto et al., 2016]

Bioassay on larvae. In order to estimate the variation in larvae susceptibility, induced by the combined treatment of permethrin and siRNA, it was necessary to perform a mortality bioassay. Briefly, following the same procedure of the previous expression bioassay, groups of 25 larvae were soaked in 178µl of siRNA or scrambled siRNA at a concentration of 0.03µg/µl and 0.06µg/µl in RNAasi free water. Five replicates were carried out for siRNA and three for scramble sequence, while six additional replicas/groups were treated only with water. After three hours of treatment each group was gently transferred into 100 ml of well water plus an LD_{50} of permethrin (0.072mg/l). Three groups of 25 larvae soaked in just well water were used as control. Mortality was assessed after 6 hours and 24 hours of permethrin exposure and larvae were considered dead if static, even after a mechanical stimulus.

Systemic RNAi effect. To verify the presence of a systemic RNAi effect with a silencing beyond the gut, the tissue in which there is the up-take of the siRNA, we soaked third instar larvae for 3 hours in the higher concentration (0.06µg/µl) of a fluorescent siRNA (alexa fluor 488). The diffusion of the fluorescent signal was analysed by Zeiss Axio Zoom.V16 stereo microscope immediately after the exposure to the siRNA and after 24 hours. Also, larvae were exposed to permethrin as before and survivors at six and 24 hours were dissected separating gut and carcass, and RNA was extracted by the carcass alone. The further steps for extraction and expression analysis were conducted as previously described for the whole larva analysis.

ABCG4 expression analysis on adults. To investigate if the down-regulation of ABCG4 persists in the adults after permethrin exposure, a specific bioassay has been performed soaking three groups of 50 third instar larvae in 357µl of 0.12µg/µl siRNA while one group of 50 non-treated larvae were used as control. After three hours, the larvae have been transferred in 70ml of well water and they were allowed to develop to the adult stage. The induction experiment has been done according the WHO protocol exposing the mosquitoes to an LD_{50} of permethrin (45µg/ml) for one hour and transferring them into clean tubes for 24 hours. Alive mosquitoes have been pooled in groups of five, collected in extraction buffer and RNA was extracted using the ReliaPrep™ RNA Tissue Miniprep System (Promega). Quantification, retro-transcription and relative expression analysis were performed as previously described for larvae.

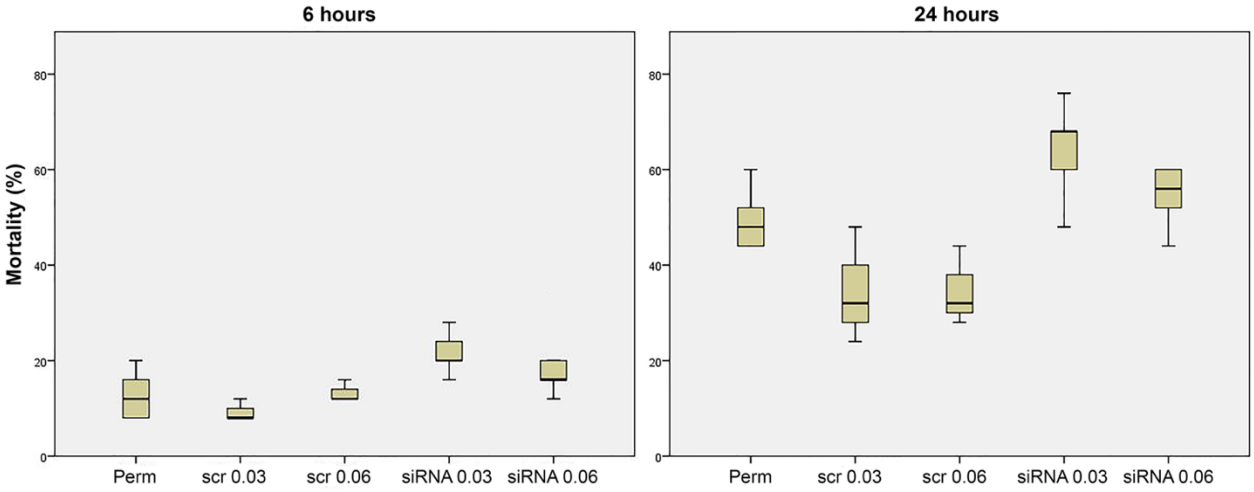
Statistical analysis. To evaluate the effect of cited treatments on larval mortality, a generalized linear mixed model (GLMM) with linear regression was implemented. The rate of larval mortality was logarithmic transformed and then introduced into the model as the dependent variable. The explanatory variables were the treatment (categorical variable) and the time of exposure (continuous variable, expressed in hours). In the model, we first included interaction between the two fixed factors; if non-significant ($P > 0.05$), the interaction was omitted from the final model. The identity of each replicate baker was included in the model as a random intercept effect.
To evaluate differences in ABCG4 gene expression levels in both whole larval bodies and carcasses of dissected larvae after gut removal exposed to permethrin, sirna 0.03 and sirna 0.06, one-way ANOVA was implemented followed by post hoc LSD test. Differences were tested at 6 and 24 hours. Statistical analyses were performed using SPSS ver. 20.0 (IBM, Chicago, IL).

RESULTS

Bioassays on larvae

The mortality data observed in bioassays well fitted the Probit dose-response model (Chi-Square probability <0.0001). The permethrin LD_{50} dose was 0.072mg/l. Mortality was assessed after six and 24 hours showing no statistical differences between the permethrin alone treatment and those with the two doses of scrambled siRNAs (0.03µg/µl and 0.06µg/µl). After six hours permethrin treatment induced a 12.8% mortality, while 21.6 and 20% mortality in response to 0.03µg/µl and 0.06µg/µl respectively. The same mortality pattern is maintained after 24 hours where permethrin exposure led to a 45.6% mortality, increased by siRNAs to 64 and 58.4% respectively (Fig.1).

Figure 1. Mortality rates after six and 24 hours of LD_{50} permethrin exposure in silenced and non-silenced larvae of *An. stephensi*.



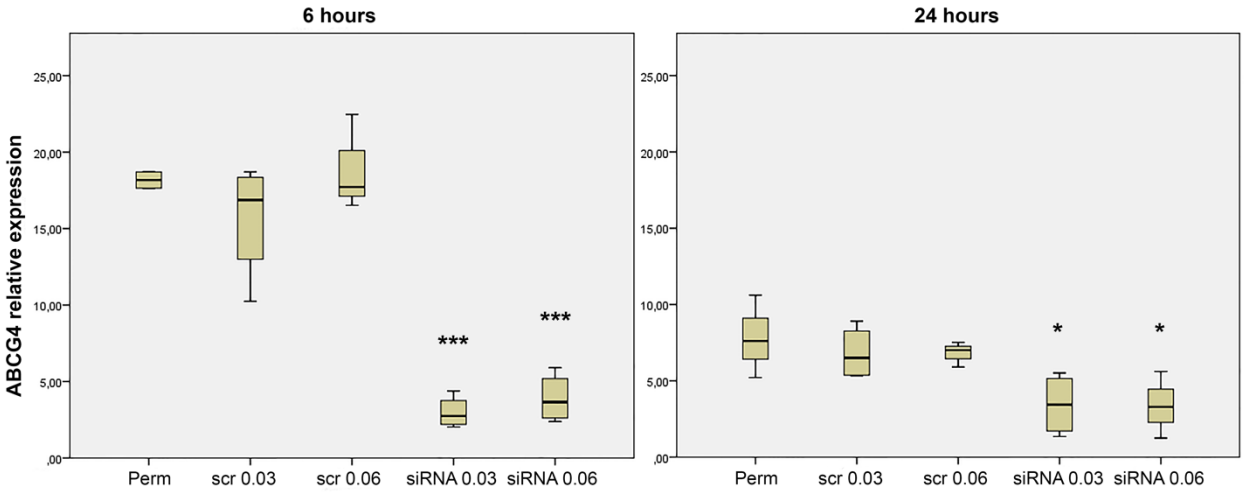
ABCG4 relative expression analysis in whole larva

Relative expression of ABC4 transporter was assessed through qRT-PCR on larvae exposed to LD_{50} of permethrin alone and in combination with siRNAs and scrambled siRNAs after six hours and 24 hours of exposure (Fig.2). After six hours, permethrin induced an up-regulation of ABCG4 of 18.17±6.64-fold, while the two siRNA concentrations in addition to the insecticide induced an up-regulation of 2.85±1.11 and 3.66±1.50-fold respectively, when compared with control. No statistically significant differences have been detected between control and treatments with siRNAs. When compared to permethrin alone, treatments in combination with siRNAs showed a relative expression of 0.16±0.06 and 0.20±0.08 respectively highlighting a down-regulation of 6.38 and 4.96-fold. Differences in relative expression in the five treatments were detected ($p<0.001$). Post hoc LSD test showed that permethrin treated larvae did not differ from those treated with scramble siRNA (both $p>0.05$), while a significant difference was detectable with the two siRNA concentrations (both<0.001). No statistically significant differences have been detected between the two concentrations of siRNA ($p>0.05$).

After 24 hours, the insecticide induced an up-regulation of 7.49±1.24-fold in permethrin treated larvae compared to the controls. Permethrin in combination with the two siRNA concentrations led to an up-regulation of 2.93±0.98 and 2.84±1.42-fold respectively for lower and higher concentration when compared to control. No statistically significant differences have been detected between control and treatments with siRNAs. When compared to permethrin treatment alone, treatments with the siRNA in addition to the insecticide showed a relative expression of 0.39±0.13 and 0.38±0.19 for the lower and higher concentrations highlighting a down-regulation of 2.55 and 2.63 when compared with insecticide treatment. Differences in relative expression in the five treatments were detected ($p<0.05$). Post hoc LSD test showed that permethrin treated larvae did not differ from those treated

with scramble siRNA (both p>0.05), while a significant difference was detectable with the two siRNA concentrations (both<0.05). No statistically significant differences have been detected between the two concentrations of siRNA (p>0.05).

Figure 2. ABCG4 induction after six and 24 hours of LD_{50} permethrin exposure in silenced and non-silenced larvae of *An. stephensi*.



Systemic RNAi effect in *An. stephensi* larvae

Third instar larvae were exposed to the 0.06µg/µl concentration of the fluorescent siRNA (alexa fuor 488) for three hours to allow them to up-take the molecule. As it is possible to notice by the images, after three hours of exposure, the siRNA is localized mainly in the gut in the central part of the larval body (Fig.3B). After the three hours of exposure, larvae were moved into fresh water for 24 hours and then analysed. In this case the fluorescent signal is detectable in all the tissues of the specimen (Fig.3C).

To confirm the effect of the siRNA beyond the gut and the existence of a systemic RNAi effect in *An. stephensi* larvae, pools of 5 larvae were dissected and the expression of ABCG4 at two time-points (six and 24 hours) in the carcass was assessed. Six hours of permethrin exposure (Fig.4) induced an ABCG4 up-regulation of 38.40±2.15-fold change in the carcass, while both siRNA concentrations reduced significantly the expression due to the insecticide respectively at 14.71±1.82 and 7.40±1.75-fold compared to control (just water) highlighting a down-regulation of 2.61 and 5.19-fold compared to permethrin alone. Relative expression of ABCG4 gene tested by one-way ANOVA varied significantly between the three tested treatments after 6 hours (p<0.001). Post hoc LSD test showed that carcasses of larvae exposed to permethrin presented higher relative expression than those exposed to siRNA 0.03 and 0.06 (both p<0.001).

After 24 hours, in the carcass (Fig.4B) the insecticide induced a 20.07±0.83 fold change up-regulation compared to the control, similarly to the insecticide with lower siRNA concentration that induced a 21.18±1.19 fold change up-regulation, while 0.06µg/µl siRNA in addition to permethrin

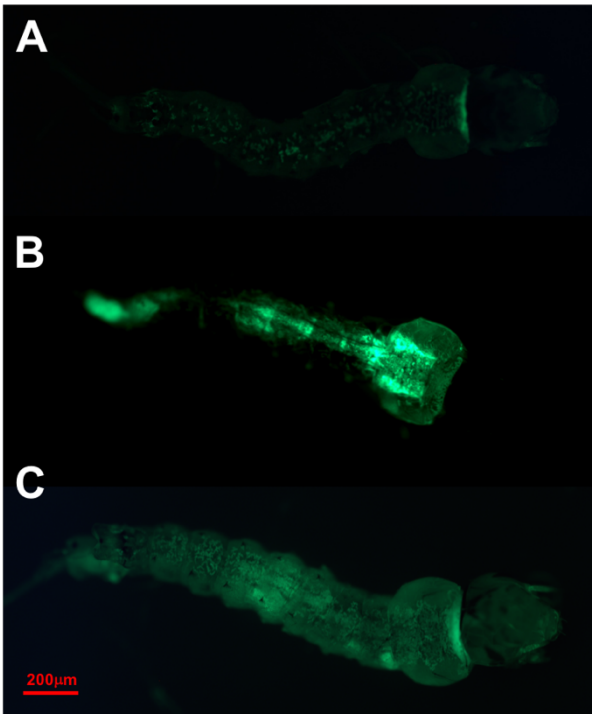
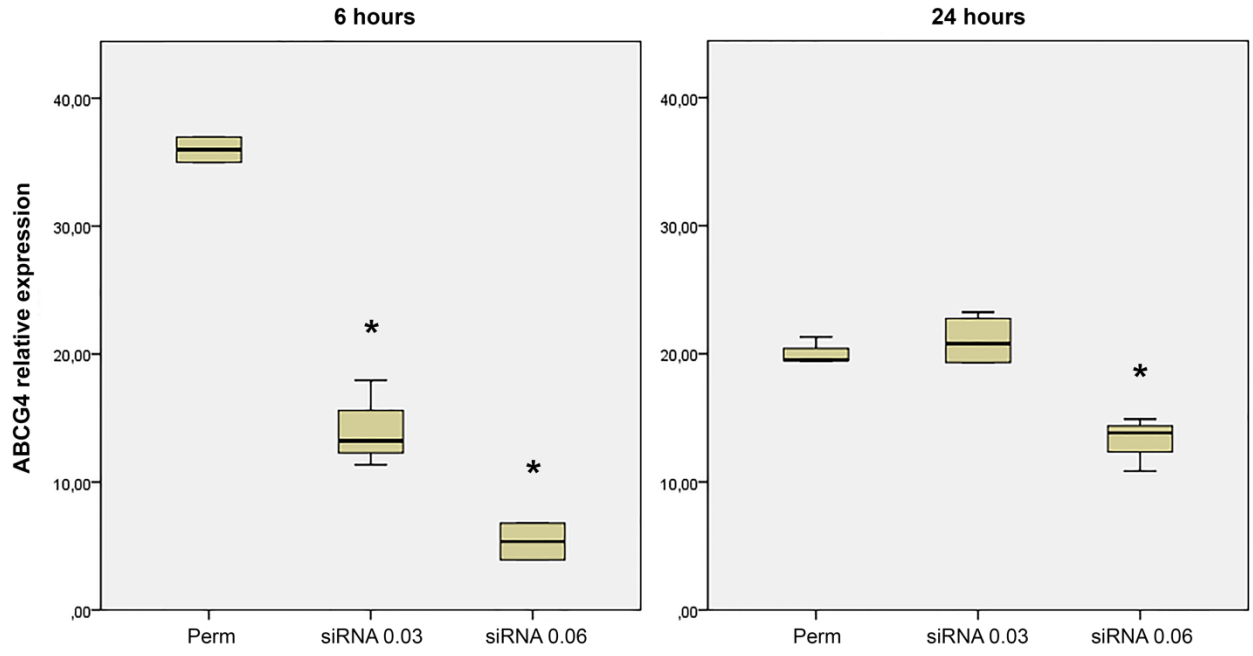


Figure 3. Third instar larvae of *An. stephensi* exposed to a 0.06mg/ml concentration of a fluorescent siRNA (alexa fluor 488). A) control larva with low auto-fluorescence. B) Larva exposed to siRNA for 3 hours and immediately analysed under the microscope. The fluorescence is concentrated in the gut. C) Larva exposed for 3 hours to the siRNA and transferred for 24 hours in water. The signal is completely diffused to the whole body.

Figure 4. Relative expression of ABCG4 in the carcass of *An. stephensi* larvae after six and 24 hours of permethrin exposure.



induced a smaller up-regulation of 13.07±1.19 fold change compared to control, but a down-regulation of 1.54-fold if compared to insecticide alone. Differences in relative expression in carcasses of the three groups of mosquitos where also detected by one-way ANOVA ($p<0.01$), but post hoc LSD test showed different trends; expression in carcasses exposed to permethrin differed only when compared with those treated with siRNA 0.06 ($p<0.01$). No differences were observed between groups treated with permethrin and siRNA 0.03 ($p>0.05$)

ABCG4 silencing in adult mosquitoes

Relative expression analysis on ABCG4 transporter gene of mosquitoes treated with permethrin was performed to evaluate if the down-regulation of the gene persisted during development from third larval stage to adulthood. ABCG4 was down-regulated by 2.29-fold (relative expression 0.43±0.12) compared to mosquitoes not treated with siRNA but just permethrin (t-student $p=0.018$) (Fig.5).

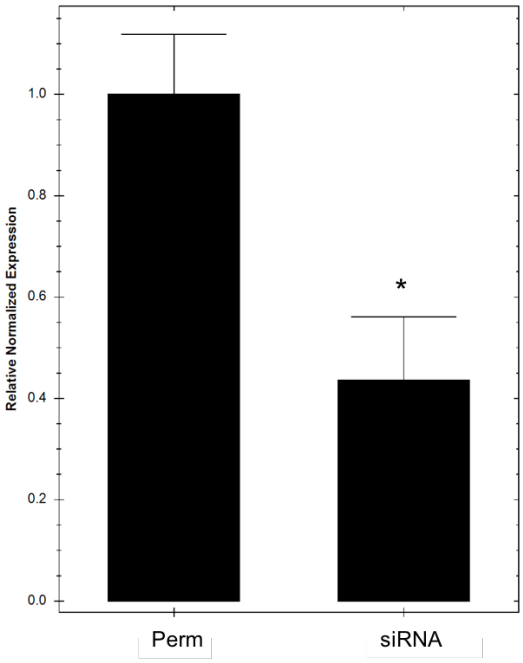


Figure 5. Relative expression of ABCG4 of silenced adults compared to non-silenced adults exposed to permethrin for one hour.

DISCUSSION

The expression analysis on the whole larval body is consistent with the data from our previous studies where we showed that the peak of over-expression of ABCG4 occurs after six hours of exposition to permethrin (Epis et al., 2014b). We have chosen this transporter as a target for silencing because it is the most up-regulated among the ABC transporters in *An. stephensi* in response to permethrin treatment demonstrating his role in the detoxification of this insecticide (Epis et al., 2014a; 2014b; De Marco et al., 2016). To assess whether the inhibition of ABCG4 has a phenotypic effect on mosquito mortality we used two siRNA concentrations. Both at 0.03µg/µl and 0.06µg/µl

concentrations, siRNAs were able to induce an increased mortality at the two time-points examined (Fig.1). This increase is correlated with the RNAi efficacy in down-regulating the expression of ABCG4 at both time points at both concentrations (Fig.2). These results are in contrast with a study of Barlett and Davis (2007) showing that the half-life of siRNA in serum varies from minutes to an hour, suggesting that only high doses of the molecule are able to reach the goal, while we were able to achieve down-regulation with 0.03µg/µl and 0.06µg/µl. In particular after 24 hours a down-regulation of around 2.5-fold compared to the insecticide alone treatment induces a mortality increase of almost twenty percentage points. Our results are consistent with studies where RNAi on a resistant strain of *Aedes aegypti* and *Culex quinquefasciatus* larvae induced an increased toxicity of temephos and permethrin (Figueira-Mansur et al., 2013; Li et al., 2014; Li et al., 2015). These data are also in accord with another study on the diamondback moth *Plutella xylostella*, where the silencing of another ABC transporter, ABCH1 (structuraly similar to ABCG sub-family members), led to larval and pupal lethal phenotypes when exposed to the Cry toxin of *Bacillus turingiensis* (Guo et al., 2015).

Since the presence of a systemic RNAi mechanisms in mosquitoes is still unclear, we decided to dissect siRNA treated and untreated larvae and to investigate the differences in ABCG4 expression and silencing in tissues beyond the gut, where the siRNA is up-taken (Singh et al., 2013). The larvae were dissected to separate the gut and malpighian tubules by the rest of the carcass to assess whether a systemic effect was detectable beyond the digestive system of the larvae. Our results show that the higher response of ABCG4 is detectable in the carcass after 6 hours of permethrin treatment. The peak of over-expression after six hours with a decrease after 24 hours (Fig.4) is consistent with previous works on the temporal response of the transporter to permethrin treatments in the whole larval body (Epis et al., 2014a; 2014b). Also, the effect of the RNAi appears to be stronger after six hours from the delivery, inducing a significant reduction in the expression of the target in the carcass for both the siRNA concentrations used. Since diptera lack the transmembrane SID-1 protein (Huvenne & Smagghe, 2010; Pillai et al., 2017) our study supports the idea of the existence of an RNAi systemic effect independent by SID-1 in accord with other studies that demonstrate a dsRNA uptake in the gut of *Ae. aegypti* and *An. gambiae* larvae and the spread of the RNAi effect to close tissues (Singh et al., 2013; Zhang et al., 2010). The relative expression of ABCG4 in response to siRNAs remains almost stable between six and 24 hours of permethrin treatment for both the concentrations used. The reduced up-regulation of ABCG4 without siRNA can explain why there are no differences between the permethrin alone treatment and the 0.03µg/µl dose. Anyhow the two concentrations seem more likely to keep the expression of the target stable during time (Fig.4) rather than continuing to down-regulate the gene of interest. A further confirm to the systemic RNAi comes from the distribution of the fluorescence associated to the siRNA in the larval body through time, showing a localization in the gut immediately after the exposure and a complete diffusion of the dye after 24 hours.

The systemic effect of RNAi in mosquitoes is confirmed also by the persistence of the down-regulation induced during the larval stage to the adulthood. In fact, third instar larvae treated with siRNA and permethrin, show an ABCG4 relative expression of 0.4 if compared to adults exposed to the insecticide alone. These data are in accord with other studies that successfully induced silencing through RNA interference in *Culex pipiens* (Lopez-Martinez et al., 2012), and *Ae. aegypti* (Whyard et al., 2015; Dalla Bona et al., 2016). In particular, Dalla Bona and colleagues tried to silence four genes through the use of dsRNA, achieving the goal just for the VGCS gene and restoring susceptibility to deltamethrin in a resistant population. Also, our results are in contrast with those obtained by Broehan and colleagues (2013) on *Tribolium castaneum* larvae where the silencing of ABCG4 led to a developmental arrest and to the death of the animals (Broehan et al., 2013). This functional discrepancy between the two species indicates a specific role of the transporter in different taxa and also exclude a major involvement of ABCG4 in the developmental process of *An. stephensi*.

CONCLUSIONS

ABCG4 down-regulation demonstrated the pivotal role of this transporter in the detoxification from permethrin in *An. stephensi* larvae, confirming to be a possible strategy for vector control. The use of a combination of RNAi and insecticide against the larval stages of this species is encouraged by these results, but further investigations are needed to verify the role of ABCG4 in detoxification in adult mosquitoes. Furthermore, we have verified the existence of a RNAi systemic effect in larvae that persist through the developmental stages to adulthood, confirming other studies in different mosquito species and contributing to clarify a mechanism still unclear and poorly studied in diptera.

REFERENCES

Alonso P.L., Tanner M. Public health challenges and prospects for malaria control and elimination. Nat Med 2013, 19(2):150-155

Anderson T.J., Nahir S., Nkhoma S., Williams J.T., Imwong M., Yi P., Socheat D., Das D., Chotivanich K., Day N.P., White N.J., Dondorp A.M. High heritability of malaria parasite clearance rate indicates a genetic basis for artemisinin resistance in western Cambodia. J Infect Dis 2010, 201(9):1326-1330

Bartlett D.W., Davis M.E. Effect of siRNA nuclease stability on the in vitro and in vivo kinetics of sirna-mediated gene silencing. Biotechnol Bioeng 2007, 97:909-921

Bretscheneider A., Heckel D.A., Vogel H. Know your ABCs: Characterization and gene expression dynamics of ABC transporters in the polyphagous herbivore *Helicoverpa armigera*. Insect Biochem

Mol Biol 2016, 72:1-9

Broehan G., Kroeger T., Lorenzen M., Merzendorfer H. Functional analysis of the ATP-binding cassette (ABC) transporter gene family of *Tribolium castaneum*. BMC Genomics 2013, 14:6

Dalla Bona A.C., Faitta Chitolina R., Lopes Fermino M., deCastro Poncio L., Weiss A., Pereira Lima J.B., Paldi N., Soares Bernardes E., Henen J., Maori E. Larval application of sodium channel homologous dsRNA restores pyrethroid insecticide susceptibility in a resistant adult mosquito population. Parasit Vectors 2016, 9:397

David J.P., Strode C., Vontas J., Nikou D., Vaughan A., Pignatelli P.M., Louis C., Hemingway J., Ranson H. The *Anopheles gambiae* detoxification chip: a highly specific microarray to study metabolic-based insecticide resistance in malaria vectors. Proc Natl Acad Sci USA 2005, 102:4080-4084

De Marco L., Sassera D., Epis S., Mastrantonio V., Ferrari M., Ricci I., Comandatore F., Bandi C., Porretta D., Urbanelli S. The choreography of the chemical defensome response to insecticide stress: insights into the *Anopheles stephensi* transcriptome using RNA-Seq. Sci Rep 2017, 7:41312

Epis S., Porretta D., Mastrantonio V., Comandatore F., Sassera D., Rossi P., Cafarchia C., Otranto D., Favia G., Genchi C., Bandi C., Urbanelli S. ABC transporters are involved in defense against permethrin insecticide in the malaria vector *Anopheles stephensi*. Parasit Vectors 2014a, 7:349

Epis S., Porretta D., Mastrantonio V., Urbanelli S., Sassera D., De Marco L., Mereghetti V., Montagna M., Ricci I., Favia G., Bandi C. Temporal dynamics of the ABC transporter response to insecticide treatment: insights from the malaria vector *Anopheles stephensi*. Sci Rep 2014b 4:7435

Fairhurst R.M., Dondorp A.M. Artemisinin-resistant *Plasmodium falciparum* malaria. Microbiol Spectr 2016, 4(3)

Figueira-Mansur J., Ferreira-Pereira A., Mansur J.F., Franco T.A., Alvarenga E.S.L., Sorgine M.H.F., Sorgine B.C., Neves B.C., Melo A.C.A., Leal W.S., Masuda H., Moreira M.F. Silencing of P-glycoprotein increases mortality in temephos-treated *Aedes aegypti* larvae. Insect Mol Biol 2013, 22(6):648-658

Guo Z., Kang S., Zhu X., Xia J., Wu Q., Wang S., Xie W., Zhang Y. The novel ABC transporter ABCH1 is a potential target for RNAi-based insect pest control and resistance management. Sci Rep 2015, 5:13728

Huvenne H., Smagghe G. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. J Insect Physiol 2010, 56:227-235

Ingham V.A., Jones C.M., Pignatelli P., Balabanidou V., Vontas J., Wagstaff S.C., Moore J.D., Ranson H. Dissecting the organ specificity of insecticide resistance candidate genes in *Anopheles gambiae*: known and novel candidate genes. BMC Genomics 2014, 15:1018

Karunamoorthi K. Vector control: a cornerstone in the malaria elimination campaign. Colin Microbiol Infect 2011, 17:1608-1616

Li T., Liu L., Zhang L., Liu N. Role of G-protein-coupled receptor-related genes in insecticide resistance of the mosquito, *Culex quinquefasciatus*. Sci Rep 2014, 4:6474

Li T., Cao C., Yang T., Zhang L., He L., Xi Z., Bian G., Liu N. A G-protein-coupled receptor regulation pathway in cytochrome P450-mediated permethrin-resistance in mosquitoes, *Culex quinquefasciatus*. Sci Rep 2015, 5:17772

Lopez-Martinez G., Meuti M., Denlinger D.L. Rehydration driven RNAi: a novel approach for effectively delivering dsRNA to mosquito larvae. J Med Entomol 2012, 49:215-218

Lumjuan N., Rajatileka S., Changsom D., Wicheer J., Leelapat P., Prapanthadara L.A., Somboon P., Lycett G., Ranson H. The role of the *Aedes aegypti* Epsilon glutathione transferases in conferring resistance to DDT and pyrethroid insecticides. Insect Biochem Mol Biol 2011, 41:203-209

Mastrantonio V., Ferrari M., Epis S., Negri A., Scucciamarra G., Montagna M., Favia G., Porretta D., Urbanelli S., Bandi C. Gene expression modulation of ABC transporter genes in response to permethrin in adults of the mosquito malaria vector *Anopheles stephensi*. Acta Tropica 2017, 171:37-43

Pillai A.B., Nagarajan U., Mitra A., Krishnan U., Rajendran S., Hoti S.L., Mishra R.K. RNA interference in mosquito: understanding immune responses, double-stranded RNA delivery systems and potential applications in vector control. Insect Mol Biol 2017, 26(2):127-139

Rooker S., Guillemaud T., Berge J., Pasteur N., Raymond M. Coamplification of esterase A and B genes as a single unit in *Culex pipiens* mosquitoes. Heredity 1996, 77:555-561

Singh A.D., Wong S., Ryan C.P., Whyard S., Oral delivery of double-stranded RNA in larvae of the yellow fever mosquito, *Aedes aegypti*: implications for pest mosquito control. J Insect Sci 2013, 13:69

Tikar S.N., Kumar A., Prasad G.B., Prakash S. Temephos-induced resistance in *Aedes aegypti* and its cross-resistance studies to certain insecticides from India. Parasites Res 2009, 105:57-63

Tikar S.N., Mendki M.J., Sharma A.K., Sukumaran D., Veer V., Prakash S., Parashar B.D. Resistance status of the malaria vector mosquitoes *Anopheles stephensi* and *Anopheles subpictus* towards adulticides and larvicides in arid and semi-arid areas of India. J Insect Sci 2011, 11:85

Tilley L., Straimer J., Gnadig N.F., Ralph S.A., Fidock D.A. Artemisinin action and resistance in *Plasmodium falciparum*. Trends Parasitol 2016, 32(9):682-696

Weill M., Berthomieu A., Berticat C., Lutfalla G., Nègre V., Pasteur N., Philips A., Leonetti J.P., Fort P., Raymond M. Insecticide resistance: a silent base prediction. Curr Biol 2004, 14(14):R552-R553

Whyard S., Erdelyan C.N., Partridge A.L., Singh A.D., Beebe N.W., Capina R. Silencing the buzz: a new approach to population suppression of mosquitoes by feeding larvae double-stranded RNAs. Parasit Vectors 2015, 8:96

World Health Organization. World Malaria Report 2017. Geneva: World Health Organization 2017. License: CC BY-NC-SA 3.0 IGO

World Health Organization. Guidelines fo laboratory and field testing for mosquito larvicides. 2005

Yamamoto D.S., Sumitani M., Kasashima K., Sezutsu H., Matsuoka H. Inhibition of malaria infection in transgenic Anopheline mosquitoes lacking salivary gland cells. PLoS Pathog 2016, 12(9):e1005872

Zhang X., Zhang J., Zhu K.Y. Chitosan/double-stranded RNA nanoparticle-mediated RNA interference to silence chitin synthase genes through larval feeding in the African malaria mosquito (*Anopheles gambiae*). Insect Mol Biol 2010, 19(5):683-693

PAPER 4:

How heterogeneous is the involvement of ABC transporters against insecticides?

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

2016; 157: 131-135 DOI:10.1016/j.actatropica.2016.02.002

How heterogeneous is the involvement of ABC transporters against insecticides?

Daniele Porretta[1], Sara Epis[2,*], Valentina Mastrantonio[1], Marco Ferrari[2], Romeo Bellini[3], Guido Favia[4], Sandra Urbanelli[1]

[1]Department of Environmental Biology, University "La Sapienza" of Rome, Italy

[2]Department of Veterinary Science and Public Health, University of Milan, Italy

[3]Agriculture Environment Centre "G. Nicoli", Crevalcore, Italy

[4]School of Bioscience and Veterinary Medicine, University of Camerino, Italy

*Corresponding author at: Department of Veterinary Science and Public Health, University of Milan, Via Celoria 10, 20133 Milan, Italy. E-mail addresses: daniele.porretta@uniroma1.it (D. Porretta)

Received 14 September 2015 Received in revised form 2 February 2016 Accepted 3 February 2016 Available online 6 February 2016

Acknowledgements

The authors are grateful to Professor Claudio Genchi and Professor Claudio Bandi, for suggestions and comments on the manuscript. This work was supported by MIUR Prin 2010-2011, grant number: 2010PJJ4W3 (to C. Genchi).

Abstract

Understanding the molecular mechanisms underlying cellular defence against xenobiotic compounds is a main research issue in medical and veterinary entomology, as insecticide/acaricide resistance is a major threat in the control of arthropods. ABC transporters are recognized as a component of the detoxifying mechanism in arthropods. We investigated the possible involvement of ABC transporters in defence to the organophosphate insecticide temephos in the malarial vector *Anopheles stephensi*. We performed bioassays on larvae of *An. stephensi*, using insecticide alone and in combination with ABC-transporter inhibitors, to assess synergism between these compounds. Next, we investigated the expression profiles of six ABC transporter genes in larvae exposed to temephos. Surprisingly, neither bioassays nor gene expression analyses provided any evidence for a major role of ABC transporters in defence against temephos in *An. stephensi*. We thus decided to review existing literature to generate a record of other studies that failed to reveal a role for ABC transporters against particular insecticides/acaricides. A review of the scientific literature led to the recovery of 569 papers about ABC transporters; among these, 50 involved arthropods, and 10 reported negative results. Our study on *An. stephensi* and accompanying literature review highlight the heterogeneity that exists in ABC transporter involvement in defence/resistance mechanisms in arthropods.

1. Introduction

Understanding the molecular mechanisms underlying cellular defence against xenobiotic compounds is a major goal of medical and veterinary entomology. In this context, the importance of ABC transporters, efflux pumps located in the cellular membrane that belong to the ATP-binding cassette (ABC) transporter family, has been increasingly recognized in recent years in a variety of organisms. The activity of ABC transporters has been correlated with resistance to cancer chemotherapy in humans, drug resistance in protozoa, antibiotic resistance in bacteria, and pesticide detoxification in nematodes and arthropods (Lage, 2003). With respect to arthropod pests, the involvement of ABC transporters in insecticide defence/resistance has been reported for agricultural pests (Podsiadlowski et al., 1998; Gaertner et al., 1998; Aurade et al., 2010) and in parasites or vectors of human and animal diseases such as ticks, mosquitoes, body lice and bed bug (Buss et al., 2002; Buss and Callaghan, 2008; Porretta et al., 2008; Pohl et al., 2011; Yoon et al., 2011; Zhu et al., 2013; Dermauw and Van Leeuwen, 2014; Cafarchia et al., 2015). The association between ABC transporters and insecticides was assessed by drug efflux, synergistic studies, measurement of ATPase activity or, more recently, by gene expression profile and gene silencing by RNA interference (Strycharz et al., 2010; Yoon et al., 2011; Figueira-Mansur et al., 2013; Zhu et al., 2013; Dermauw and Van Leeuwen, 2014).

ABC transporters are highly polyspecific as they act against many toxic compounds that are structurally and chemically different, and against all most classes of insecticides (Buss and Callaghan, 2008; Dermauw and Van Leeuwen, 2014). Mutations in the ABCC2 transporter gene have been showed to be involved in resistance to Bacillus thuringiensis (Bt) Cry1A toxin as well, which led to hypothesize a central role of this transporter in the mode of action of Bt toxins (reviewed in Heckel, 2012).

In our previous studies, using ABC transporter inhibitors in combination with gene expression analyses, we showed that ABC transporters are involved in defence against the pyrethroid insecticide permethrin in larvae of a susceptible strain of the mosquito *Anopheles stephensi* (Epis et al., 2014a, b), the major vector of human malaria in the Middle, East, and Southeast Asia (Sinka et al., 2011). Bioassays with permethrin alone and in combination with the ABC inhibitor verapamil showed that the inhibition of ABC transporters increased the permethrin toxicity of about 5-fold (SF = 5.48). Likewise, gene expression analyses of six ABC transporter genes (AnstABCB2, AnstABCB3, AnstABCB4, AnstABCBmember6, AnstABCG4 and AnstABCC11) isolated in *An. stephensi*, showed significant increased expression of the AnstABCBmember6 and AnstABCG4 genes in larvae exposed at permethrin (Epis et al., 2014a, b). In this paper, our first aim was to investigate whether ABC transporters are involved in defence against the organophosphate insecticide temephos in this species, as observed in other mosquitoes, such as *Aedes caspius*

(Porretta et al., 2008) and *Aedes aegypti* (Figueira-Mansur et al., 2013). Bioassays using inhibitors of ABC transporters were performed in combination with gene expression analyses following Epis et al. (2014a, b). Since we did not find evidence for a major role for ABC transporters in *An. stephensi*, our second aim was to review the scientific literature in order to identify previous studies that have yielded negative results in arthropods.

2. Materials and methods

Bioassays and molecular analyses were performed using a susceptible strain of *An. stephensi* (Liston) available from the insectary of the University of Camerino, that has been used in our previous works about ABC transporters (Epis et al., 2014a, b). Eggs were put into spring water and the hatched larvae were maintained in spring water at 28 ± 1 ° C and 85–90% relative humidity with photoperiods (12:12 L-D) and fed daily with fish food (Tetra, Melle, Germany).

The bioassays were conducted on third instar larvae, according to standard protocols as described in Epis et al. (2014a); using temephos insecticide (PESTANAL, C21H20Cl2O3, Sigma-Aldrich S.r.l., Milan, Italy). Briefly, groups of 25 larvae were put in test vials (250-mL plastic glasses) with 100 mL of spring water and different concentrations of insecticide alone or plus a sub-lethal dose of the inhibitor verapamil or Cyclosporine A (CsA) (Sigma-Aldrich, Milano, Italy). Two additional groups of larvae, treated only with water and acetone (the solvent of temephos) were used as controls. Mortality was assessed at 24 h post-treatment and all tests were performed in quadruple. The sub-lethal doses of inhibitors were obtained by bioassays as described above and in Epis et al. (2014a). Larval mortality data were subjected to probit regression analysis as implemented in the Polo Plus software (Robertson et al., 2003) to estimate the LD_{50} values with their 95% confidence intervals (95% CI). The occurrence of significant differences between the treatment with temephos alone and temephos plus inhibitor was investigated (i) by testing the hypotheses of equality (equal slopes and intercepts) of the regression lines from the treatments and (ii) by estimating the synergistic ratio between the LD_{50} of the treatments (SR = LD_{50} temephos/LD_{50} temephos + inhibitor) and its 95% CI.

The expression profiles of the six ABC transporter genes isolated in *An. stephensi* and previously analysed for their involvement in defence against permethrin (AnstABCB2, AnstABCB3, AnstABCB4, AnstABCBmember6, AnstABCG4 and AnstABCC11) were analysed in larvae exposed to the LD_{50} dose of temephos (0.0096 mg/L as estimated by the bioassays above). Two pools of ten larvae were collected at two times after treatment (6 and 24 h). Controls (water plus acetone) were collected at the same times. To check for consistency, the experiment was repeated twice. Every pool of larvae was stored in RNAlater (Qiagen, Hilden, Germany) until molecular analysis. Protocols for RNA extraction, cDNAs synthesis and quantitative RT-PCRs as well as primer pairs used are described in detail in Epis et al. (2014a, b). Significant differences in expression between treated

and control groups have been evaluated as implemented in the CFX Manager software (Bio-Rad).

In order to find cases of none or poor association between ABC transporters and insecticides (see Section 3), a revision of the scientific literature about the association between ABC transporters and pesticides was performed in the bibliographic database Scopus as follow: keywords “ABC-transporters AND pesticides”; search options: “all fields”; “article”; “published from all years to present”; “life sciences”. The search was updated on the 1st March 2015. The papers found were triangulated against the most recent review about ABC transporters in arthropods (Dermauw and Van Leeuwen, 2014). We selected all papers where association between ABC transporters and insecticides was assessed by drug efflux; synergistic studies; measurement of ATPase activity or gene expression profile; following (Dermauw and Van Leeuwen, 2014). A “negative result” was registered when none or poor association between ABC transporters and insecticides were observed in susceptible and/or resistant strain.

3. Results and discussion

If ABC transporters play a major role in defence against temephos in *An. stephensi*, a higher larval mortality rate would be expected in bioassays using temephos in combination with ABC inhibitors than in bioassays using insecticide alone. Likewise, increased expression of one or more ABC transporter genes could be predicted in larvae exposed to temephos (Buss and Callaghan, 2008; Dermauw and Van Leeuwen, 2014; Epis et al., 2014a). The results obtained from the bioassays and from gene expression analyses here reported failed to confirm these expectations.

The results of toxicity assays are reported in Table 1 and Fig. 1. The mortality data obtained from all bioassays fitted the probit dose-response model (Chi-squared tests $P > 0.05$). The verapamil sub-lethal dose was 100 mM, which confirms the results observed by Epis et al. (2014a) while the CsA sub-lethal dose was 120 mM. No significant difference in toxicity was observed between treatments, and hypotheses of regression line equality from the treatments with temephos and temephos plus inhibitors were not rejected either for verapamil (Chi-squared value: 3.95, degrees of freedom = 2, $P > 0.139$) or for CsA (Chi-squared value: 4.13, degrees of freedom = 2, $P > 0.126$). Similarly, the Synergic Ratio (SR) between the LD_{50} of the treatments was 1.138 (95% CI 0.992–1.306) and 1.074 (95% CI 0.941–1.227) for verapamil and CsA, respectively. In both cases, the 95% CI of SR includes a value of 1, which shows that the LDs were not significantly different (Robertson et al., 2003). The results of quantitative RT-PCR analysis are shown in Table 2. In treated larvae, moderate but not statistically significant up-regulation was observed only for the AnstABCB2 gene (2.29 ± 0.29 and 1.6 ± 0.12 after 6 h of treatment with temephos, and 1.75 ± 1.08 and 1.32 ± 0.23 after 24 h of treatment) and ABCG4 gene (1.06 ± 0.29 after 24 h of treatment). Taken together, the above results provide evidence that ABC transporters don’t play a major role in defence against temephos in *An. stephensi* larvae. We are aware that bioassays and gene expression analyses using individuals from

different strains or natural populations could led to different results as well as that the six ABC transporter loci, analysed in this study, are only a part of the *An. stephensi* ABC transporter genes potentially involved in defence against insecticides. However, using the same experimental design, we have previously shown positive evidence for the involvement of ABC transporters in defence against permethrin in the same strain of *An. stephensi*, so the negative results presented here are informative of differences in the response between the two insecticides.

Table 1 Toxicity of temephos and temephos in conjunction with verapamil and CsA in 3th instar larvae of *Anopheles stephensi*. Intercept and slope of the regression line of the treatments estimated by probit analysis are shown. LD_{50}: Letal Dose at 50%; SR: Synergic Ratio; 95% CI: 95% Confidence Intervals.

Insecticide	Intercept (±SE)	Slope (±SE)	LD_{50} (95% CI)	SR (95% CI)
Temephos	-3.397 (0.211)	3.453 (0.209)	0.0096mg/L (0.0081-0.0116)	
Temephos + verapamil (100μM)	-3.099 (0.191)	3.341 (0.204)	0.0085mg/L (0.0073-0.0099)	1.138 (0.992-1.306)
Temephos + CsA (120μM)	-2.866 (0.173)	3.009 (0.170)	0.0089mg/L (0.0078-0.0102)	1.074 (0.941-1.227)

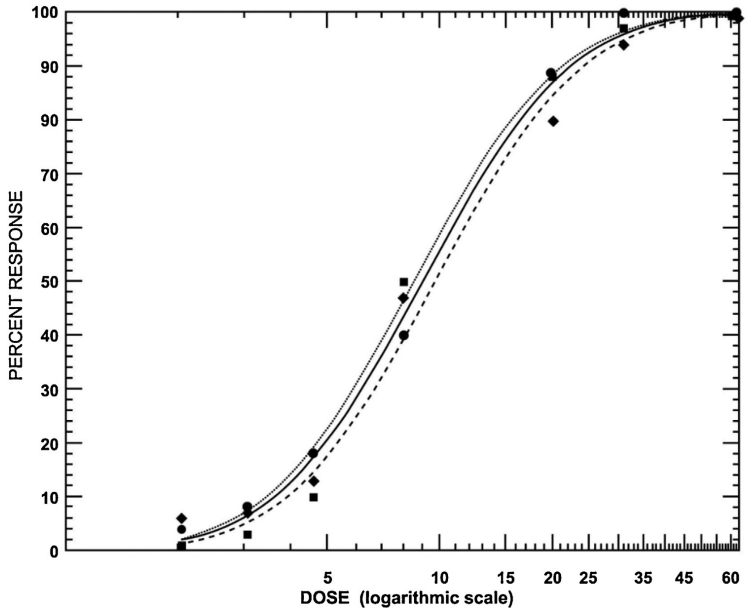


Fig. 1 Larval mortality × log(dose) plots and regression lines for bioassays. Temephos alone (diamonds, continuous line); temephos in conjunction with verapamil (100 μM) (black circles, dotted line); temephos in conjunction with Cyclosporine A (120 μM) (squares, discontinued line). In the temephos alone bioassays, seven insecticide concentrations were used (0.00204, 0.00306, 0.00459, 0.008, 0.02, 0.031, 0.062 mg/L) to have mortality in the range 1–99%. The same concentrations were used in the bioassays with temephos in combination with sub-lethal dose of verapamil or CsA. The verapamil sub-lethal dose was determined by a bioassay with ten different concentrations of verapamil from 20 to 560 μM; the CsA sub-lethal dose was determined by a bioassay with eight different concentrations from 5 to 200 μM.

Table 3 List of the papers where no association between ABC transporters and insecticides was found. For each paper we reported also the evidences of positive association when they were found. SP: synthetic pyrethroids; OP: organophosphates; MLs: macrocyclic lactones. S: susceptible stains; R: resistant strains. (−) no association; (+) association.

Species	Insecticide	Negative association	Positive association		References
Lepeophtheirus salmonis	avermectin emamectin benzoate (EMB)	no verapamil synergism in S adult no differences in expression of four MPRs genes between S and R; no increased expression of four MPRs in S and R exposed to EMB	CsA synergism in R and S; verapamil synergism in S	(−) S; (+) R (−) S; (−) R	Heumann et al. (2012)
Anopheles gambiae s.l.	deltamethrin (SP)	no verapamil synergism in R adult females		(−) R	Chouaïbou et al. (2014)
Caligus rogercresseyi	deltamethrin(SP)	no over Cr-Pgp expression (qPCR) in S adult males exposed to deltamethrin	over Cr-Pgp expression (qPCR) in S adult females exposed to deltamethrin	(+)♂;(−)♀	Valenzuela-Muñ ozetal.(2014)
Liposcelis bostrychophila	deltamethrin (SP) malathion (OP)	no over expression of ABC-transporters (RNA-seq) in S adult exposed to deltamethrin and malathion		(−) S	Dou et al. (2013)
Drosophila melanogaster	DDT (Organochlo-rines)	no verapamil synergism in DDT-susceptible 91-R strain	verapamil synergism in DDT-resistant 91-R strain	(−)S;(+)R	Strycharz et al. (2013)
Bemisia tabaci	thiametoxam (Neonicotinoids)	no over expression of ABCG gene (qPCR) in egg and nymph stages of R strain	over expression of ABCG gene (qPCR) in adult stages of R strain	differences between life-cycle stages	Yang et al. (2013)
Rhipicephalus (Boophilus) microplus	ivermectin (MLs) abamectin (MLs) moxidectin (MLs) chlorpyriphos (OP)	no CsA and MK571 synergism in S larvae for all insecticides	CsA and MK571 synergism in R larvae for all insecticides	(−)S;(+)R	Pohl et al. (2012)
Rhipicephalus (Boophilus) microplus	cypermetrin (SP) amitraz	no CsA and MK571 synergism in S and R larvae for both toxic		(−)S;(−)R	Pohl et al. (2012)
Rhipicephalus (Boophilus) microplus	ivermectin (MLs)	no CsA and MK571 synergism in S larvae no CsA and MK571 synergism in S adult female no significant increase in the transcription level of RmABCB10 in IVM-susceptible females exposed to IVM	CsA and MK571 synergism in R larvae CsA and MK571 synergism in R adult females significant increase in the transcription level of RmABCB10 in IVM-resistant females exposed to IVM	(−)S;(+)R	Pohl et al. (2011)
Helicoverpa armigera	cypermethrin (SP) fenvalerate (SP) endosulfan (OP) monocrotophos (OP) quinolphos (OP)	no increased P-gp expression (Western Blot) in susceptible larvae for all insecticides	increased P-gp expression (Western Blot) in multiresistant larvae for all insecticides	(−) S; (+) R	Srinivas et al. (2004)
Culex pipiens	chlorpyrifos (OP) endosulfan (OP) ivermectin (MLs) cypermethrin (SP)	no verapamil synergism in S and R 4th instar larvae with chlorpyrifos	verapamil synergism in S and R 4th instar larvae with endosulfan, ivermectin, cypermethrin	(−) S; (−) R	Buss et al. (2002)

The case study of *An. stephensi*/temephos is not unique in arthropods. In our review of the scientific literature, we analysed 569 papers concerning the association between ABC transporters and insecticides. We found 50 papers involving arthropod species and, 10 of these reported negative results (Table 3).

About 40 years of research on mammalian ABC transporters have revealed their central role in protecting tissues from exogenous and endogenous toxins. However, several questions have not been satisfactorily answered (Sharom, 2008), and these gaps in our knowledge are larger in arthropods, in which ABC transporters research is a relatively new field (82% of reviewed papers were published in the last five years, data not shown). Until now, the interest in ABC transporters in arthropods has focused on their involvement in insecticide defence and/or resistance. As a consequence, more emphasis has been placed on positive results than on negative ones (notably, negative results are mostly embedded in studies where positive results are also described). If we consider these cases in the light of the positive results available in the literature, it may be observed that (i) ABC transporters in different species may or may not be involved in defence against the same insecticide in susceptible strains. For example, they are involved against temephos in *Ae. caspius* (Porretta et al., 2008) and *Ae. aegypti* (Figueira-Mansur et al., 2013), but not in *An. stephensi*. (ii) ABC transporters in the same species may or may not be involved in defence against different insecticides as observed in the mosquito *Culex pipiens* (Table 3). (iii) Differences in the same species between the sexes or life-stages against the same insecticide can occur (such as the sea lice *Caligus rogercresseyi*, Table 3). Lastly, (iv) susceptible and resistant strains of the same species may behave in the same way against the same insecticide (i.e., ABC transporters may be involved in neither or both) or differently (i.e., the ABC transporters are involved in resistant but not in susceptible strains). Therefore, these observations highlight the high heterogeneity that exists in ABC transporter involvement in defence/resistance mechanisms in arthropods and may form the basis for future studies.

Studies aimed at the understanding of the mechanisms of substrate binding and transport as well as of the relationships between ABC transporters and other detoxifying enzymatic systems are urgently needed, since they act against synthetic insecticides that are currently used as well as biological as Bt toxins as recently emerged (Heckel 2012) and are likely contributing to the evolution of resistances. We argue that negative results can contribute to these research issues by using them as “blank”. It may be speculated, for example, that in the case of *An. stephensi*, comparative transcriptomic analyses in larvae exposed to permethrin (against which the involvement of ABC transporters has been reported (Epis et al., 2014a) and temephos (no involvement, this study) could provide information about the regulatory factors underlying the interaction between ABC transporters and other detoxifying enzymes.

References

Aurade, R.M., Jayalak shim, S.K., Sreeramulu, K., 2010. P-Glycoprotein ATPase from the resistan pest, *Helicoverpa armigera*: purification, characterization and effect of various insecticides on its transport function. Biochim. Biophys. Acta 1798, 1135–1143.

Buss, D.S., Callaghan, A., 2008. Interaction of pesticides with p-glycoprotein and other ABC proteins: a survey of the possible importance to insecticide: herbicide and fungicide resistance. Pestic. Biochem. Physiol. 90, 141–153.

Buss, D.S., McCaffery, A.R., Callaghan, A., 2002. Evidence for p-glycoprotein modification of insecticide toxicity in mosquitoes of the *Culex pipiens* complex. Med. Vet. Entomol. 16, 218–222.

Cafarchia, C., Porretta, D., Mastrantonio, V., Epis, S., Sassera, D., Iatta, R., Immediato, D., Ramos, R.A.N., Lia, R.P., Dantas-Torres, F., Urbanelli, S., Otranto, D., 2015. Potential role of ATP-binding cassette transporters against acaricides in the brown dog tick *Rhipicephalus sanguineus* sensu lato. Med. Vet. Entomol. 29, 88–93.

Chouaïbou, M., Bingham Zivanovic, G., Knox, T.B., Pates Jamet, H., Bonfoh, B., 2014. Synergist bioassays: a simple method for initial metabolic resistance investigation of field *Anopheles gambiae* s.l. populations. Acta Trop. 130, 108–111.

Dermauw, W., Van Leeuwen, T., 2014. The ABC gene family in arthropods: comparative genomics and role in insecticide transport and resistance. Insect Biochem. Mol. Biol. 45, 89–110.

Dou, W., Shen, G.M., Niu, J.Z., Ding, T.B., Wei, D.D., Wang, J.J., 2013. Mining genes involved in insecticide resistance of *Liposcelis bostrychophila* Badonnel by transcriptome and expression profile analysis. PLoS One 8, e79878.

Epis, S., Porretta, D., Mastrantonio, V., Urbanelli, S., Sassera, D., De Marco, L., Mereghetti, V., Montagna, M., Ricci, I., Favia, G., Bandi, C., 2014a. ABC transporters are involved in defense against permethrin insecticide in the malaria vector *Anopheles stephensi*. Parasites Vectors 7, 349.

Epis, S., Porretta, D., Mastrantonio, V., Comandatore, F., Sassera, D., Rossi, P., Favia, G., Bandi, C., Urbanelli, S., 2014b. Temporal dynamics of the ABC transporter response to insecticide treatment: insights from the malaria vector *Anopheles stephensi*. Sci. Rep. 4, 7435.

Figueira-Mansur, J., Ferreira-Pereira, A., Mansur, J.F., Franco, T.A., Alvarenga, E.S., Sorgine, M.H., Neves, B.C., Melo, A.C., Leal, W.S., Masuda, H., Moreira, M.F., 2013. Silencing of P-glycoprotein increases mortality in temephos-treated *Aedes aegypti* larvae. Insect Mol. Biol. 22 (6), 648–658.

Gaertner, L.S., Murray, C.L., Morris, C.E., 1998. Transepithelial transport of nicotine and vinblastine

in isolated malpighian tubules of the tobacco hornworm (*Manduca sexta*) suggests a P-glycoprotein-like mechanism. J. Exp. Biol. 201 (18), 2637–2645.

Heckel, D.G., 2012. Learning the ABCs of Bt: aBC transporters and insect resistance to *Bacillus thuringiensis* provide clues to a crucial step in toxin mode of action. Pestic. Biochem. Physiol. 104, 103–110.

Heumann, J., Carmichael, S., Bron, J.E., Tildesley, A., Sturm, A., 2012. Molecular cloning and characterisation of a novel P-glycoprotein in the salmon louse *Lepeophtheirus salmonis*. Comp. Biochem. Physiol. C 155, 98–205.

Lage, H., 2003. ABC-transporters: implications on drug resistance from microorganisms to human cancers. Int. J. Antimicrob. Agents 22, 188–199.

Podsiadlowski, L., Matha, V., Vilcinskas, A., 1998. Detection of a P-glycoprotein related pump in Chironomus larvae and its inhibition by verapamil and cyclosporin A. Comp. Biochem. Physiol. B 121, 443–450.

Pohl, P.C., Klafke, G.M., Carvalho, D.D., Martins, J.R., Daffre, S., da Silva Vaz Jr., I., Masuda, A., 2011. ABC transporter efflux pumps: a defence mechanism against ivermectin in *Rhipicephalus (Boophilus) microplus*. Int. J. Parasitol. 41, 1323–1333.

Pohl, P.C., Klafke, G., Júnior, J., Martins, J., Silva Vaz Jr., I., Masuda, A., 2012. ABC transporters as a multidrug detoxification mechanism in *Rhipicephalus (Boophilus) microplus*. Parasitol. Res. 111, 2345–2351.

Porretta, D., Gargani, M., Bellini, R., Medici, A., Punelli, F., Urbanelli, S., 2008. Defence mechanism against insecticides temephos and diflubenzuron in the mosquito *Aedes caspius*: the P-glycoprotein efflux pumps. Med. Vet. Entomol. 22, 48–54.

Robertson, J.A., Preisler, H.K., Russell, R.M., 2003. Polo Plus: Probit and Logit Analysis. LeOra Software, Petaluma, CA.

Sharom, F.J., 2008. ABC multidrug transporters: structure, function and role in chemoresistance. Pharmacogenomics 9 (1), 105–127.

Sinka, M.E., Bangs, M.J., Manguin, S., Chareonviriyaphap, C., Patil, A.P., Temperley, W.H., Gething, P.W., Elyazar, I.R.F., Kabaria, C.W., Harbach, R.E., Hay, S.I., 2011. The dominant Anopheles vectors of human malaria in Asia–Pacific: occurrence data, distribution maps and bionomic précis. Parasites Vectors 4, 89.

Srinivas, R., Udikeri, S.S., Jayalakshmi, S.K., Sreeramulu, K., 2004. Identification of factors

responsible for insecticide resistance in *Helicoverpa armigera*. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 137, 261–269.

Strycharz, J.P., Lao, A., Li, H., Qiu, X., Lee, S.H., Sun, W., Yoon, K.S., Doherty, J.J., Pittendrigh, B.R., Clark, J.M., 2013. Resistance in the highly DDT-resistant 91-R strain of *Drosophila melanogaster* involves decreased penetration increased metabolism, and direct excretion. Pestic. Biochem. Physiol. 107, 207–217.

Valenzuela-Mun˜oz, V., Nun˜ez-Acun˜a, G., Gallardo-Escárate, C., 2014. Molecular characterization and transcription analysis of P-glycoprotein gene from the salmon louse *Caligus rogercresseyi*. J. Aquacult. Res. Dev. 5, 4.

Yang, N., Xie, W., Jones, C.M., Bass, C., Jiao, X., Yang, X., Liu, B., Li, R., Zhang, Y., 2013. Transcriptome profiling of the whitefly *Bemisia tabaci* reveals stage-specific gene expression signatures for thiamethoxam resistance. Insect Mol. Biol. 22, 485–496.

Yoon, K.S., Strycharz, J.P., Baek, J.H., Sun, W., Kim, J.H., Kang, J.S., Pittendrigh, B.R., Lee, S.H., Clark, J.M., 2011. Brief exposures of human body lice to sublethal amounts of ivermectin over-transcribes detoxification genes involved in tolerance. Insect Mol. Biol. 20 (6), 687–699.

Zhu, F., Gujar, H., Gordon, J.R., Haynes, K.F., Potter, M.F., Palli, S.R., 2013. Bed bugs evolved unique adaptive strategy to resist pyrethroid insecticides. Sci. Rep. 3, 1456.

PAPER 5:

ABC transporters are not involved in the detoxification of *Azadirachta indica* extracts in *Anopheles stephensi* larvae

APJTD
Asian Pacific Journal of Tropical Disease

In submission

ABC transporters are not involved in the detoxification of *Azadirachta indica* extracts in *Anopheles stephensi* larvae

Ferrari M.[1,*], Negri A.[2], Romeo C.[3], Varotto Boccazzi I.[1], Nodari R.[1], Habluetzel A.[4], Molteni G.[5], Corbett Y.[1]

[1]Department of Biosciences, University of Milan, Italy
[2]Department of Environmental Biology, University "La Sapienza" of Rome, Italy
[3]Department of Veterinary Medicine, University of Milan, Italy
[4]School of Pharmacy, University of Camerino, Italy
[5]Department of Chemistry, University of Milan, Italy

*Corresponding author at: Department of Biosciences, University of Milan, Via Celoria 26, 20133 Milan, Italy.
E-mail addresses: marco.ferrari@unimi.it (Ferrari M.)

Funding support: The work was supported by grants from the Italian Ministry of Education, University and Research (MIUR) (Prin 2012 protocol 2012T85B3R)

Keywords
mosquito defences, natural insecticides, neem tree, vector control, detoxification

Abstract

Objective: detoxifying pathways of mosquitoes against neem (*Azadirachta indica*) extracts are still unclear. The aim of the present study is to investigate the role of ABC-transporters in this process in *Anopheles stephensi,* one of the main malaria vectors in southern Asia.

Methods: third instar larvae of *An. stephens*i were fed with fish food alone or in combination with neem extract at 0.5, 1, 5 and 10%. Six ABC-transporter genes from three different subfamilies (B, C and G) have been analysed to assess relative expression compared to the control. A bioassay was also performed to assess larval mortality rate at the different concentrations in combination with verapamil, an ABC-transporter inhibitor.

Results: No significant variation in the expression levels of any transporter belonging to the B, C and G subfamilies was detected. Furthermore, the use of verapamil, an ABC transporter inhibitor, did not induce an increase of mortality at any of the tested neem extract doses.

Conclusion: ABC transporters are not involved in response/defence to neem extracts in *An. stephensi* larvae.

Introduction

Malaria is one of the main problems in developing countries. About 216 million cases occurred in 2016, with 445000 deaths [1]. The use of long lasting insecticidal nets (LLINs), artemisinin-based therapies and indoor residual spraying (IRS) are the main interventions aimed to prevent malaria infections and spread. Vector control through insecticides is a core component of malaria control programmes, but the continuous use of chemical compounds led to resistance insurgence in different vector populations that threaten the global malaria control efforts [2, 3, 4]. Of the 73 malaria endemic countries providing data to the WHO, 60 reported resistance to at least one insecticide class, while 50 reported resistance to two or three classes [1]. For this reason, new modalities of insecticides are needed. Botanical sources represent a promise alternative to search for insecticide activity. *Azadirachta indica*, commonly known as neem, has been used for centuries in traditional medicine [5, 6]. This was probably due to the wide effects that this plant has on parasites and other agents of infection [6]. *A. Indica* and other Meliaceae species have shown a strong larvicidal, anti-emergence, repellency, and anti-oviposition effect in different mosquito species [7- 14]. In addition, products based on neem rarely induce resistance thanks to their wide mode of action [15]. The main active substance of neem-based insecticides is the molecule azadirachtin [16], that induces a mitochondria-mediated apoptosis in lepidopteran cells [17] and the blockage of calcium channels [18]. It is now known that part of the detoxification process against xenobiotics is mediated by ATP-Binding Cassette (ABC) transporters in different mosquito species such as *An. stephensi* [19-23], *An. gambiae* [24], *Aedes aegypti* [25], *Ae. Albopictus* [26]. In particular, on *An. stephensi* it has been demonstrated that, among the eight sub-families of ABC transporters existing in insects, the B and G sub-families play a major role in the detoxification against permethrin, showing a pattern of response that varies with time [19, 20, 22, 23]. Despite their importance against pyrethroids, these genes are not differentially expressed in response to larval exposure to temephos, a widely used larvicide, highlighting an insecticide-specific involvement of the transporters in this mosquito species [21]. For these reasons, the goal of our study is to thoroughly investigate the implication of ABC-transporters in *An. stephensi* defence against neem.

Matherial and methods

Bioassay

All mosquitoes used in this study derived from a susceptible *An. stephensi* Liston colony of the insectary of the University of Camerino, Italy, maintained at standard conditions (28±1°C, 85% humidity, 12:12 L-D) and fed with fish food (FF) (Tetra, Melle, Germany). Third instar larvae were used for bioassays and molecular analysis, as described in Epis et al. [19, 20]. Experimental groups

were fed with FF containing neem seed extract (FF + neem) at different concentrations: 0.5, 1, 5 and 10%. To obtain these concentrations, *A. indica* seeds have been crushed and homogenized to 1g FF in 50 ml chloroform (Sigma-Aldrich), mixed for 10 minutes and then evaporated at reduced pressure (37°C, 3 mmHg) with a Büchi R 200 rotavapor. The powder obtained was left at room temperature for 24h.

For the bioassay, five groups of 25 third instar larvae were put in 100 ml of spring water and fed with FF + neem at different concentrations (0, 0.5, 5 and 10%), alone or in combination with a sub-lethal dose of the inhibitor verapamil (100 □M), as reported in previous works [19, 20]. Verapamil is a blocker of calcium channels that competes with toxic compounds for the extrusion by transmembrane pumps [27]. Control groups with FF alone or verapamil with FF were included. Mortality was assessed every 24h for three days.

To investigate the effect of different treatments on the larval mortality, we run a Generalized Linear Mixed Model (GLMM) with Poisson error structure, using the number of dead larvae as dependent variable and considering replicates as a residual-type random component. We explored the effect on the response variable of dose/concentration of insecticide (i.e. 0%, 0.5%, 1%, 5%, 10%), addition of verapamil, time of treatments (24, 48 or 72h) and verapamil dose. The initial number of larvae of each replicate was included in the model as a covariate. Interactions were excluded from the final model when not significant. Interpretation of effects with more than two levels was based on pair-wise t-tests of Differences of Least Square Means (DLSM), applying Tukey correction for multiple comparisons. The analysis were carried out through PROC GLIMMIX in SAS/STAT9.4 software (Copyright © 2002-2012, SAS Institute Inc., Cary, NC, USA).

Gene Expression profile after insecticide treatment

The six genes analysed, encoding for ABC transporters (*Anst*ABCB2, *Anst*ABCB3, *Anst*ABCB4, *Anst*ABCBmember6, *Anst*ABCG4, *Anst*ABCC11) in *An. stephensi*, were chosen due to their involvement in the defence against the insecticide permethrin. The expression profile of these genes was evaluated in larvae after 0.5, 24, 48 and 72h of treatment at different neem concentration. Table 1 shows the primers used in this work. RNA extraction, cDNA synthesis, quantitative RT-PCRs were performed following the protocol described in detail in [19, 20]. Two different genes, RPS7 and GAPDH, have been used as reference genes to normalize relative expression. To detect any significant effect of neem treatment on the expression of ABC genes, RT-PCR data were analysed trough non-parametric Wilcoxon two-sample tests, due to the non-normal distribution of some samples [28]. For each of the six genes and each of the dose-time combinations, differences in ΔCT (CT_{target} - $CT_{housekeeping}$) between treated and control (i.e. dose 0) samples were compared. Estimates of ΔΔCT values and their 95% confidence limits were obtained through the Hodges-Lehman method. All the analysis were carried out using PROC NPAR1WAY in SAS® 9.4 Software (Copyright © 2012 SAS Institute Inc., Cary, NC, USA).

Table 1 Primer sequences of ABC transporters and housekeeping genes of *Anopheles stephensi*.

Gene	Forward primer	Reverse primer	bp	Source
*Anst*ABCB2	TATCAAGTTCACGGATGTAGAGT	TATCCACCTTGCCACTGTC	185	[19]
*Anst*ABCB3	CAACCGTTCCGTAATACTACC	ACTGGTAGCCCAATGTGAAG	133	[19]
*Anst*ABCB4	GGACAAAACATTCGGGAGG	CGTAGTGAATGTTGTGGCG	109	[19]
*Anst*ABCBmember6	CTGGAGACGCTGAGAGATA	TACTCCTCGGTGAACTGG	125	[19]
*Anst*ABCC11	GGTTGGATTGGCTTTCGTG	ATAACCGACTCCCGTTTCG	156	[20]
*Anst*ABCG4	ATGAGCCCATTCGTCCTG	AGCGTGGAGAAGAAGCAG	158	[19]
RPS7	AGCAGCAGCAGCACTTGATTTG	TAAACGGCTTTCTGCGTCACCC	90	[35]
GAPDH	GCCGTCGGCAAGGTCATCCC	TTCATCGGTCCGTTGGCGGC	166	[36]

Results

Statistical analysis of bioassay data revealed that mortality of larvae increased significantly with time ($F_{2,18}$=41.4; p<0.0001) and at higher doses/concentrations of insecticide ($F_{4,36}$=16.8; p<0.0001). However, addition of verapamil had no effect on larvae mortality, either as a single factor or in interaction with insecticide (both p>0.13). Furthermore, the analysis of RT-PCR data (tab.2) did not reveal any effect of neem treatment on ABC genes expression: ΔCT values of treated samples were not significantly different from controls, for any of the 6 target genes and any of the dose-time combinations (all p>0.05).

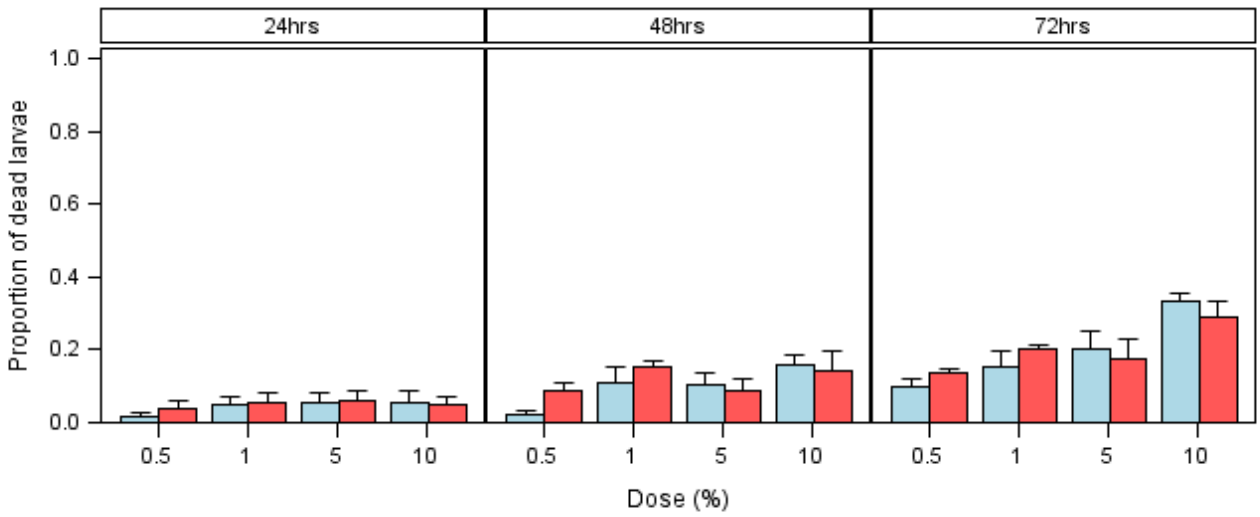


Figure 1 Proportion of dead larvae at different times and different insecticide concentrations, with (white bars) and without (grey bars) Verapamil addition. Error bars indicate standard errors.

Table 2 Relative expression of *Anopheles stephensi* ABC genes obtained with quantitative RT-PCR after treatment with neem extract at different times. Expression level of the control, non-treated larvae, was considered to be the basal level (equal 1). RPS7 and GAPDH were used as reference genes to normalize expression levels. The values are expressed as mean ± standard error.

Exposure time	Insecticide concentration	*Anst*ABCB2	*Anst*ABCB3	*Anst*ABCB4	*Anst*ABCB member6	*Anst*ABCC11	*Anst*ABCG4
0.5h	0.5%	1,43 ± 0,11	1,37 ± 0,19	1,39 ± 0,35	1,39 ± 0,16	1,16 ± 0,09	1,28 ± 0,70
	1%	0,98 ± 0,29	0,78 ± 0,27	1,13 ± 0.39	0,78 ± 0,28	0,93 ± 0,21	1,46 ± 0,92
	5%	1,95 ± 0,70	1,29 ± 0,51	1,11 ± 0,45	1,32 ± 0,45	0,77 ± 0,18	1,02 ± 0,79
	10%	2,74 ± 0.57	1,66 ± 0,36	1,21 ± 0,22	2,08 ± 0,44	0,96 ± 0,13	1,98 ± 1,42
24 h	0.5%	1,25 ± 0,08	1,29 ± 0,14	1,31 ± 0,18	1,32 ± 0,20	0,95 ± 0,04	1,06 ± 0,31
	1%	1,28 ± 0,09	1,47 ± 0,26	1,39 ± 0,32	1,32 ± 0,28	1,12 ± 0,07	2,26 ± 0,93
	5%	0,81 ± 0,09	0,55 ± 0,08	0,66 ± 0,07	0,98 ± 0,16	0,95 ± 0,13	1,62 ± 0,61
	10%	1,15 ± 0,14	0,87 ± 027	1,11 ± 0,15	1,06 ± 0,24	1,07 ± 0,15	1,55 ± 0,46
48 h	0.5%	0,62 ± 0,26	0,59 ± 0,13	0,47 ± 0,10	0,66 ± 0,19	1,25 ± 0,24	0,80 ± 0,36
	1%	1,05 ± 0,27	0,79 ± 0,12	0,76 ± 0,10	0,85 ± 0,22	1,13 ± 0,06	1,42 ± 0,73
	5%	0,87 ± 0,22	0,92 ± 0,35	0,67 ± 0,19	0,85 ± 0,18	0,88 ± 0,14	2,55 ± 1,06
	10%	1,13 ± 0,60	0,50 ± 0,12	0,46 ± 0,03	0,54 ± 0,16	0,97 ± 0,03	2,24 ± 1,60
72 h	0.5%	0,49 ± 0,06	0,57 ± 0,13	0,88 ± 0,10	0,82 ± 0,09	0,86 ± 0,10	1,35 ± 0,21
	1%	0,50 ± 0,27	0,58 ± 0,20	0,80 ± 0,08	0,88 ± 0,21	0,93 ± 0,04	2,13 ± 0,31
	5%	0,55 ± 0,01	0,59 ± 0,19	1,02 ± 0,03	0,93 ± 0,20	0,59 ± 0,09	2,23 ± 0,33
	10%	0,73 ± 0,10	0,61 ± 0,14	1,13 ± 0,11	1,05 ± 0,23	0,84 ± 0,04	2,55 ± 0,37

Discussion

A. indica has been used for centuries in traditional medicine and as a natural insecticide, and the effects of azadirachtin has been demonstrated in Hymenoptera like *Bombus terrestris* and *Habrobracon hebetor* [29-30], Coleoptera like *Zabrotes subfasciatus* [31], Lepidoptera like *Tuta absoluta* [32], Hemiptera like *Aphis gossypii* [33] and several Diptera species [7-14]. Several works focus on the effects of natural neem extracts and commercial neem-based products on mosquitoes because of their medical and veterinary importance, and for the insurgence of insecticide resistance among vector populations. To assess whether mosquitos respond to neem extracts activating the same detoxification pathways they use against other insecticides, we used a bioassay and molecular analysis approach. As expected consulting previous works on neem extract effects on mosquito larvae [7, 8, 11, 27, 34], the bioassay confirms that the tested compound has dose- and time-dependent larvicidal effect on *An. stephensi* larvae. At the same time, the results of insecticide exposure in combination with the ABC transporter inhibitor, verapamil, demonstrate that ABC transporters are not involved in the cellular response of *An. stephensi* to neem extracts, in contrast with the results of other insecticide tests [19] in which verapamil, added in combination with permethrin treatment, induced an increased mortality compared to the insecticide alone. However, this outcome is consistent with data reported by Porretta and colleagues [21], who tested the insecticide temephos against *An. stephensi*. In this work they showed that verapamil was not able

to increase larval mortality. Also, the expression of the ABC transporters in our study is maintained at basal levels despite the insecticide treatment, in accord with Porretta [21] but in contrast with other works on *An. stephensi* exposed to permethrin that induced a response of the transporters varying with time [19, 20, 23]. These results indicate that different compounds can induce different responses of the mosquito ABC transporters. This work cannot exclude the implication of other detoxification mechanisms and, for this reason, further investigations are needed to clarify and amplify the set of transporters analysed, taking into account also different metabolic pathways that could be involved. Nevertheless, these results are important in an attempt of widen the global knowledge on the detoxification from xenobiotics in the mosquito *An. stephensi*.

Conflict of interest statement

The authors declare not to have any financial or personal conflict of interest.

Acknowledgements

We would like to thank prof. Sandra Urbanelli, Daniele Porretta and Valentina Mastrantonio for the help and the suggestions, and also for reviewing the manuscript.

References

1. World Health Organization. World Malaria Report 2017. Geneva: World Health Organization 2017. License: CC BY-NC-SA 3.0 IGO
2. Tikar SN, Mendki MJ, Sharma AK, Sukumaran D, Veer V, Prakash S, Parashar BD. Resistance status of the malaria vector mosquitoes, *Anopheles stephensi* and *Anopheles subpictus* towards adulticides and larvicides in arid and semi-arid areas of India. J Insect Sci 2011; 11:85-94
3. Vontas J, Kioulos E, Palvidi N, Morou E, della Torre A, Ranson H. Insecticide resistance in the major dengue vectors *Aedes albopictus* and *Aedes aegypti*. Pestic Biochem Physiol 2012; 104:126-131
4. Alonso PL, Tanner M. Public health challenges and prospects for malaria control and elimination. Nat Med 2013; 19(2):150-155
5. Omar S, Zhang J, MacKinnon S, Leaman D, Durst T, Philogene BJ, et al. Traditionally-used antimalarials from the Meliaceae. Curr Top Med Chem 2003; 3:133-139
6. Soh PN, Benoit-Vical F. Are West African plants a source of future antimalarial drugs? J Ethnopharmacol 2007; 114:130-140
7. Zebitz CPW. Effects of some crude and azadirachtin enriched neem *Azadirachta indica* seed kernel extracts on larvae of *Aedes aegypti.* Entomol Exp Appl 1984; 35:11-14
8. Zebitz CPW. Effects of tree neem seed kernel extracts and azadirachtin on larvae of different mosquito species. J App Entomol 1986; 102:455-463
9. Murugan K, Jeyabalan D. Mosquitocidal effect of certain plants extracts on *Anopheles stephensi*. Curr Sci 1999; 76:631-633
10. Su T, Mulla MR. Oviposition bioassay responses of *Culex tarsalis* and *Culex quinquefasciatus* to neem products containing azadirachtin. Entomol Exp Appl 1999; 91:337-345
11. Vatandoost H, Vaziri VM. Larvicidal activity of a neem tree extract (Neemarin) against mosquito larvae in the Islamic Republic of Iran. East Mediterr Health J 2004; 10(4-5):573-581
12. Wandscheer CB, Duque JE, da Silva MAN, FukuyamaY, Wohlke JL, Adelmann J, Fontana JD. Larvicidal action of ethanolic extracts from fruit endocarps of *Melia azedarach* and *Azadirachta indica* against the dengue mosquito *Aedes aegypti*. Toxicon 2004; 44(8):829-835
13. Nathan SS, Savitha G, George DK, Narmadha A, Suganya L, Chung PG. Efficacy of *Melia azedarach* L. Extract of the malaria vector *Anopheles stephensi* Liston (Diptera: Culicidae). Bioresour Technol 2006; 97(11):1316-1323

14. Dembo EG, Abay S, Dahiya N, Ogboi JS, Christophides GK, Lupidi G, et al. Impact of repeated NeemAzal-treated blood meals on the fitness of *Anopheles stephensi* mosquitoes. ParasitVectors 2015; 8:94-106
15. Benelli G, Murugan K, Panneerselvam C, Madhiyazhagan P, Conti B, Nicoletti M. Old ingredients for a new recipe? Neem cake, a low-cost botanical by-product in the fight against mosquito-borne diseases. Parasitol Res 2015; 114:391-397
16. Siegwart M, Graillot B, Blachere Lopez C, Besse S, Bardin M, Nicot PC, et al. Resistance to bio-insecticides or how to enhance their sustainability: a review. Front Plant Sci 2015; 6:381
17. Huang J, Lu C, Hu M, Zhong G. The mitochondria-mediated apoptosis of lepidopteran cells induced by azadirachtine. PLoS ONE 2013; 8:e58499
18. Qiao J, Zou X, Lai D, Yan Y, Wang Q, Li W, et al. Azadirachtin blocks the calcium channel and modulates the cholinergic miniature synaptic current in the central nervous system of *Drosophila*. Pest Manag Sci 2014; 70:1041-1047
19. Epis S, Porretta D, Mastrantonio V, Comandatore F, Sassera D, Rossi P, et al. ABC transporters are involved in defense against permethrin insecticide in the malaria vector *Anopheles stephensi*. Parasit Vectors 2014; 7:349-355
20. Epis S, Porretta D, Mastrantonio V, Urbanelli S, Sassera D, DeMarco L, et al. Temporal dynamics of the ABC transporter response to insecticide treatment: insights from the malaria vector *Anopheles stephensi*. Sci Rep 2014; 4:7435
21. Porretta D, Epis S, Mastrantonio V, Ferrari M, Bellini R, Favia G, Urbanelli S. How heterogeneous is the involvement of ABC transporters against insecticides? Acta Trop 2016; 157:131-135
22. Mastrantonio V, Ferrari M, Epis S, Negri A, Scuccimarra G, Montagna M, Favia G, et al. Gene expression modulation of ABC transporter genes in response to permethrin in adults of the mosquito malaria vector *Anopheles stephensi.* Acta Trop 2017; 171:37-43
23. De Marco L, Sassera D, Epis S, Mastrantonio V, Ferrari M, Ricci I, et al. The choreography of the chemical defensome response to insecticide stress: insights into the *Anopheles stephensi* transcriptome using RNA-Seq. Sci Rep 2017; 7:41312
24. Nkya TE, Akhouayri I, Poupardin R, Batengana B, Mosha F, Magesa S, et al. Insecticide resistance mechanisms associated with different environments in the malaria vector *Anopheles gambiae*: a case study in Tanzania. Malar J 2014; 13:28-42
25. Bariami V, Jones CM, Poupardin R, Vontas J, Ranson H. Gene amplification, ABC transporters and cytochrome P450s: unravelling the molecular basis of pyrethroid resistance in the dengue vector, *Aedes aegypti*. PLoS Negl Trop Dis 2012; 6(6):e1692
26. Esquivel CJ, Cassone BJ, Piermarini PM. A de novo transcriptome of the Malpighian tubules in non-blood-fed and blood-fed Asian tiger mosquitoes *Aedes albopictus*: insights into diuresis, detoxification, and blood meal processing. Peer J 2016; 4:e1784

27. Thomas H, Coley HM. Overcoming multidrug resistance in cancer: an update on the clinical strategy of inhibiting P-glycoprotein. Cancer Control 2003; 10(2):159-165
28. Yuan JS, Reed A, Chen F, Stewart CN. Statistical analysis of real-time PCR data. BMC Bioinform 2006; 7:85-96
29. Barbosa WF, De Meyer L, Guedes RNC, Smagghe G. Lethal and sublethal effects of azadirachtin on the bumblebee *Bombus terrestris* (Hymenoptera: Apidae). Ecotoxicology 2015; 24(1):130-142
30. Abedi Z, Saber M, Gharekhani G, Mehrvar A, Kamita SG. Azadirachtin and Cypermethrin on *Habrobracon hebetor* (Hymenoptera: Braconidae). J Econ Entomol 2014; 107(2):638-645
31. Vilca Mallqui KS, Vieira JL, Guedes RNC, Gontijo LM. Azadirachtin-induced hormesis mediating shift in fecundity-longevity trade-off in the Mexican Bean Weevil (Chrysomelidae: bruchinae). J Econ Entomol 2014; 107(2):860-866
32. Tomé HVV, Martins JC, Correa AS, Galdino TVS, Picanço MC, Guedes RNC. Azadirachtin avoidance by larvae and adult females of the tomato leafminer *Tuta absoluta*. Crop Protection 2013; 46:63-69
33. Yi F, Zou C, Hu Q, Hu M. The joint action of destruxins and botanical insecticides (rotenone, azadirachtin and paeonolum) against the Cotton Aphid, *Aphis gossypii* Glover. Molecules 2012; 17(6):7533-7542
34. Dua VK, Pandey AC, Raghavendra K, Gupta A, Sharma T, Dash AP. Larvicidal activity of neem oil (*Azadirachta indica*) formulation against mosquitoes. Malar J 2009; 8:124-129
35. Capone A, Ricci I, Damiani C, Mosca M, Rossi P, Scuppa P et al. Interactions between Asaia, Plasmodium and Anopheles: new insights into mosquito symbiosis and implications in malaria symbiotic control. Parasit Vectors 2013; 6:182-194
36. Yamamoto DS, Sumitani M, Kasashima K, Sezutsu H, Matsuoka H. Inhibition of Malaria infection in transgenic Anopheline mosquitoes lacking salivary gland cells. PLoS Pathog 2016; 12(9):e1005872

CONCLUSIONS

The importance of ABC transporters

The focus of this thesis is the detoxification mechanisms of the mosquito *An. stephensi*, the main urban malaria vector in the south of Asia, against different classes of insecticides. The study was carried out on a laboratory sensitive strain because data were still missing on this species and also an attempt to clarify the relation between ABC transporters and other detoxifying enzymes was needed. Furthermore, these data will be the base for further studies that will allow to identify constitutively differentially expressed genes in resistant *An. stephensi* strains through a RNA-seq approach. The first paper contributes to clarify the temporal response of several detoxification enzymes (CYPs, CCEs, UGTs, GSTs and ABCs) in larvae exposed to permethrin, the major representative of the pyrethroids, one of the main insecticide classes used for vector control. The results confirm the implication of GSTs and CYPs as seen on *An. stephensi* wild resistant populations from Afghanistan (Safi et al., 2017). However, this is the first study that aim to analyse the whole arthropod defensome through a transcriptomic approach not only in a single time-point, but all along the insecticide exposure time. Also, this paper confirms, through a transcriptomic approach, the role of ABC transporters, with four of them up-regulated in at least one time-point, and previously identified through a RT-qPCR approach on the same species (Epis et al., 2014a, b). These proteins are pivotal in the earlier phase of detoxification (phase 0) to decrease the intracellular concentration of the toxicant and, in the last phase (phase III) to extrude the catabolites of the degradation of the same xenobiotic.

Both in larvae and adults, some of the genes analysed were up-regulated (B and G sub-families), while some were down-regulated at different time-points indicating a complex response of the defensome and a reallocation of depleted resources due to the metabolic costs imposed by the insecticide. This response is indeed stage-, time- and sex-dependent, with a different pattern of up- and down-regulation in larvae and adults, males and females, and at different time-points after xenobiotic exposure. These findings are particularly interesting for vector control, since the presence of the same inducible genes in all the developmental stages of a sensitive mosquito strain increases the risk of resistance insurgence by point mutations, duplications or mutations in regulatory regions (Le Goff et al., 2006; Liu, 2015; Nkya et al., 2013).

From these analysis, one particular transporter, ABCG4, has been identified as a main actor in the response to permethrin. It has been possible to demonstrate its importance in *An. stephensi* larvae through RNAi technique, showing that the silencing of this transporter induces an increased larval mortality in accordance with other studies on different species (Guo et al., 2015). These data are important in a view of mosquito control and fight to the insecticide resistance mechanisms, providing a tool for restoring chemical efficacy also in area with highly resistant populations.

The heterogeneity in the response of ABC transporters

As previously written, these papers highlight the heterogeneity in the response of these proteins (Porretta et al., 2016; Ferrari et al., submitted. See articles 4 and 5), according to the sex of the specimens and to the developmental stage (Mastrantonio et al., 2017; Ferrari et al., submitted. See articles 2 and 3). But more interestingly, also the class of chemical used influences the expression of ABC transporters. Temephos and a natural extract of *A. indica* seeds failed to induce a response of the genes analysed indicating a specificity in the detoxification role of the ABC transporters. In these last two papers, we investigated the role of just six transporters that we knew to be involved against permethrin, but other transporters of different sub-families could be differentially expressed in response to these insecticides, even if the not different larval mortality with the addition of verapamil indicates that they don't have a major role in detoxification against temephos and neem extracts. Differences in the involvement of ABC transporters can be due to the insecticide class, the insect species, the life stage, the sex or the resistance status of the specimens. Anyhow, also these results are important in the understanding of detoxification mechanisms and in the wider view of vector control, in this context, it is pivotal the knowledge of the vector biology before improving any control strategy.

Future development of the project

Malaria eradication, for a malaria free world, is a reachable achievement that is, unfortunately, threatened by the insurgence of genetic and metabolic resistance both in the parasite and in the vector. Also, the lack in the knowledge of the vector biology is an obstacle to an effective management of the control efforts, exposing the future generations to the risk of malaria recrudescence episodes. For these reasons, further investigations are required for a deeper understanding of the processes on which insecticide detoxification relies and for the identification of new targets for the development of different compounds to add to the ones already in use. In the meanwhile, entomologists and molecular biologist are experimenting new methods to integrate to the old ones in the fight against the parasites and the vectors. In particular, techniques based on molecular and biotechnological approaches are keeping the attention of scientists and, among these, RNA interference and CRISPR/Cas9 editing play a major role. These methods are nowadays essential tools for investigating gene function, but also for the development of field strategies to increase insecticide effectiveness or to act as eco-friendly insecticides themselves targeting essential species-specific genes or disrupting metabolic pathways.

Several points remain unclear for the effective use of RNAi for large-scale mosquito control (Pillai et al. 2017). First of all, a delivery system able to protect the siRNA from degradation due to nucleases and pH variations naturally occurring in the environment. Some authors are trying to face this

problem including the oligonucleotides into nanoparticles (Zhang et al., 2010; Phanse et al., 2015; Das et al., 2015; Kumar et al., 2016), with the aim of increasing the amount of siRNA ingested and the silencing achieved in mosquito larvae. Another problem is the uncertainty about the presence of systemic RNAi effect in mosquitoes. Although this study, in accord with few others, seems to demonstrate a SID-1 (Systemic RNA interference defective protein 1) independent systemic RNAi, the precise mechanism of this effect is still unknown in mosquitoes and could be different depending of the species in exam. Moreover, a siRNA dose optimization is required for better results in mosquito mortality. All these points should converge on an easy, effective and not expensive method that could be applied in Third World countries, in particular in those areas that require special attention because of the presence of resistant vector populations.

For these reasons, the future aims of the project in which this thesis is included consist in the optimisation of siRNA delivery procedures and in the clarification and characterisation of systemic RNAi in *An. stephensi*. The development of new economical strategies suitable for gene silencing in the field will also be investigated.

Furthermore, the induction of the same ABC genes in all the developmental stages of sensitive *An. stephensi* mosquitoes in response to permethrin exposure and the indication that ABCs are involved in pyrethroid-resistant strains of *An. gambiae* (Pignatelli et al., 2018) constitute the base for the prosecution of this project in the attempt of investigate the role of these transporters also in *An. stephensi* resistant strains through a comparative transcriptomic approach based on the data obtained in this thesis.

References

Das S., Debnath N., Cui Y., Unrine J., Palli S.R. Chitosan, carbon quantum dot, and silica nanoparticle mediated dsRNA delivery for gene silencing in *Aedes aegypti*: a comparative analysis. ACS Appl Mater Interfaces 2015, 7:19530

Epis, S., Porretta, D., Mastrantonio, V., Comandatore, F., Sassera, D., Rossi, P., Cafarchia, C., Otranto, D., Favia, G., Genchi, C., Bandi, C., Urbanelli, S. ABC transporters are involved in defense against permethrin insecticide in the malaria vector *Anopheles stephensi*. Parasit Vectors 2014a, 7:349

Epis, S., Porretta, D., Mastrantonio, V., Urbanelli, S., Sassera, D., De Marco, L., Mereghetti, V., Montagna, M., Ricci, I., Favia, G., Bandi, C., Temporal dynamics of the ABC transporter response

to insecticide treatment: insights from the malaria vector *Anopheles stephensi*. Sci Rep 2014b, 4:7435

Guo Z., Kang S., Zhu X., Xia J., Wu Q., Wang S., Xie W., Zhang Y. The novel ABC transporter ABCH1 is a potential target for RNAi-based insect pest control and resistance management. Sci Rep 2015, 5:13728

Kumar R.D., Kumar S.P., Gandhi M.R., Al-Dhabi N.A., Paulraj M.G., Ignacimuthu S. Delivery of chitosan/dsRNA nanoparticles for silencing of wing development vestigial (vg) gene in *Aedes aegypti* mosquitoes. Int J Biol Macromol 2016, 86:89

Liu N. Insecticide resistance in mosquitoes: impact, mechanisms, and research directions. Annu Rev Entomol 2015, 60:537

Le Goff G., Hilliou F., Siegfried B.D., Boundy S., Wajnberg E., Sofer L., Audant P., ffrench-Constant, R.H., Feyereisen, R. Xenobiotic response in *Drosophila melanogaster*: sex dependence of P450 and GST gene induction. Insect Biochem. Mol. Biol. 2006, 36(8):674

Nkya T.E., Akhouayri I., Kisinza W., David J.P. Impact of environment on mosquito response to pyrethroid insecticides: facts, evidences and prospects. Insect Biochem Mol Biol 2013, 43:407

Phanse Y., Dunphy B.M., Perry J.L., Airs P.M., Paquette C.C., Carlson J.O., Xu J., Luft J.C., DeSimone J.M., Beaty B.J., Bartholomay L.C. Biodistribution and toxicity studies of PRINT hydrogel nanoparticles in mosquito larvae and cells. PLoS Negl Trop Dis 2015, 9:e0003735.

Pignatelli P., Ingham V.A., Balabanidou V., Vontas J., Lycett G., Ranson H. The Anopheles gambiae ATP-binding cassette transporter family: phylogenetic analysis and tissue localization provide clues on function and role in insecticide resistance. Insect Mol Biol 2018, 27(1):110

Pillai B.A., Nagarajan U., Mitra A., Krishnan U., Rajendran S., Hoti S.L., Mishra R.K. RNA interference in mosquito: understanding immune response, double-stranded RNA delivery systems and potential applications in vector control. Insect Mol Biol 2017, 26(2):127

Safi N.H.Z., Ahmadi A.A., Nahzat S., Ziapour S.P., Nikookar S.H., Fazeli-Dinan M., Enayati A., Hemingway J. Evidence of metabolic mechanisms playing a role in multiple insecticides resistance in *Anopheles stephensi* populations from Afghanistan. Malar J 2017, 16:100

Zhang X., Zhang J., Zhu, K.Y. Chitosan/double-stranded RNA nanoparticle-mediated RNA interference to silence chitin synthase genes through larval feeding in the African malaria mosquito (*Anopheles gambiae*). Insect Mol Biol 2010, 19:683

PAPER 1:

Supplemental matherial

Table S1. Analysis of diving and feeding activities in *Anopheles stephensi* larvae. Diving is expressed as mean number of diving/larva within 5 minutes of observations. Feeding is expressed as the mean time of feeding/larva within 5 minutes of observations. Standard deviations are shown in brackets. Ten larvae were used in each treatment. Tests and controls refer to *An. stephensi* larvae exposed to permethrin for six, 24 and 48 hours. Letters refer to Student-t test significance: equal letters refer to no significant differences ($P > 0.05$); different letters, refer to significant differences ($P < 0.05$).

	6 hours		24 hours		48 hours	
	Tests	**Controls**	**Tests**	**Controls**	**Tests**	**Controls**
Diving (n/larva)	0.2^{a} (0.45)	1.5^{b}(0.58)	0.067^{a} (0.26)	2.06^{b} (1.24)	0.33^{a} (0.49)	1.3^{b}(0.78)
Feeding (sec./larva)	14.2^{A} (19.47)	66^{B} (36.26)	5.67^{A} (9.82)	150.3^{B} (99.53)	10.93^{A} (15.14)	115.56^{B} (56.864)

Table S2. Overview of *Anopheles stephensi* cDNA libraries sequenced. The accession summaries for each cDNA library are also shown. All libraries belong to the project classified in EBI Short Read Archive as PRJEB14386 (ERP016027).

Sample	Condition	Time	Number of reads	Reads mapped (%)	Sample accession	Secondary accession	Sample unique name
T1-6h	Permethrin treatment	6h	8,426,192	84.59	ERS1203190	SAMEA4032080	Aste_permethrin1_6
T2-6h	Permethrin treatment	6h	12,427,928	83.79	ERS1203191	SAMEA4032081	Aste_permethrin2_6
T3-6h	Permethrin treatment	6h	8,075,568	84.21	ERS1203192	SAMEA4032082	Aste_permethrin3_6
C1-6h	control	6h	20,424,530	87.25	ERS1203181	SAMEA4032071	Aste_control1_6
C2-6h	control	6h	19,186,242	87.46	ERS1203182	SAMEA4032072	Aste_control2_6
C3-6h	control	6h	19,525,044	87.24	ERS1203183	SAMEA4032073	Aste_control3_6
T1-24h	Permethrin treatment	24h	12,169,006	86.74	ERS1203193	SAMEA4032083	Aste_permethrin1_24
T2-24h	Permethrin treatment	24h	17,871,396	86.42	ERS1203194	SAMEA4032084	Aste_permethrin2_24
T3-24h	Permethrin treatment	24h	12,079,144	85.13	ERS1203195	SAMEA4032085	Aste_permethrin3_24
C1-24h	control	24h	14,718,886	89.09	ERS1203184	SAMEA4032074	Aste_control1_24
C2-24h	control	24h	11,084,542	84.5	ERS1203185	SAMEA4032075	Aste_control2_24
C3-24h	control	24h	24,048,844	86.87	ERS1203186	SAMEA4032076	Aste_control3_24
T1-48h	Permethrin treatment	48h	22,551,016	87.11	ERS1203196	SAMEA4032086	Aste_permethrin1_48
T2-48h	Permethrin treatment	48h	26,742,198	86.39	ERS1203197	SAMEA4032087	Aste_permethrin2_48
C1-48h	control	48h	20,156,386	87.62	ERS1203187	SAMEA4032077	Aste_control1_48
C2-48h	control	48h	18,498,212	86.53	ERS1203188	SAMEA4032078	Aste_control2_48
C3-48h	control	48h	13,272,258	86.19	ERS1203189	SAMEA4032079	Aste_control3_48

Table S3. List of defensome genes and their expression trend after six, 24 and 48 hours of permethrin exposure. Expression trend at six, 24 and 48 hours time points is encoded as: -1, down-regulated; 0 no differential expression; 1, up-regulated (i.e. -1,-1,-1 means that the gene is down-regulated after six, 24 and 48 hours of exposure). Genes encoding for Heat shock Proteins and Cuticular Proteins are also shown.

An. stephensi ID	Expression Trend	6 hours		24 hours		48 hours		Mosquito ID	Species	Annotation	e-value	KOG ID	KEGG ID
		log_2FC	*P*	log_2FC	*P*	log_2FC	*P*						
Phase 0/III													
ASTEI02233-RA	-1,-1,-1	-1.234	0.000	-1.300	0.007	-1.218	0.000	AGAP005639-PA	*An. gambiae*	ATP-binding cassette, subfamily B (MDR/TAP), member 1	0	KOG0055	K05658
ASTEI10271-RA	-1,-1,-1	-1.897	0.000	-1.353	0.000	-1.763	0.000	AGAP006427-PA	*An. gambiae*	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	4E-102	KOG0054	NA
ASTEI05285-RA	0,-1,-1	-0.651	0.000	-1.136	0.000	-1.448	0.000	AGAP008436-PA	*An. gambiae*	ATP-binding cassette, sub-family C (CFTR/MRP), member 11	0	KOG0054	NA
ASTEI05286-RA	0,-1,-1	-0.256	0.027	-1.435	0.000	-2.029	0.000	AAEL005043-PA	*Ae. aegypti*	ATP-dependent bile acid permease	0	KOG0054	K05665
ASTEI08906-RA	0,-1,0	-0.512	0.020	-1.040	0.000	-0.374	0.144	AGAP006273-PA	*An. gambiae*	ATP-binding cassette, subfamily B (MDR/TAP), member 8	0	KOG0058	K056559
ASTEI06528-RA	0,-1,0	-0.306	0.104	-1.255	2.20e-13	-0.694	0.0003	ASTEI06528-RA	*Ae. aegypti*	Multidrug resistance protein 2 (ATP-binding cassette protein c)	0	KOG0054	K05665
ASTEI00096-RA	0,0,0	-0.545	0.056	-0.092	0.799	0.050	0.890	AGAP001777-PA	*An. gambiae*	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	1E-71	NA	NA
ASTEI04513-RA	0,0,0	0.098	0.884	-0.464	0.465	-1.118	0.060	AGAP000506-PA	*An. gambiae*	ATP-binding cassette, sub-family G (WHITE), member 2b	0	KOG0061	NA
ASTEI07694-RA	0,0,0	-0.377	0.035	-0.439	0.013	-0.591	0.001	AGAP007917-PA	*An. gambiae*	ATP-binding cassette, subfamily C (CFTR/MRP), member 10	0	KOG0054	K05674
ASTEI09515-RA	0,0,0	-0.333	0.093	-0.277	0.187	-0.001	0.997	AGAP003221-PA	*An. gambiae*	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	0	KOG0054	K05673
ASTEI09638-RA	0,0,0	0.042	0.785	-0.677	0.000	-0.380	0.005	AGAP009471-PA	*An. gambiae*	ATP-binding cassette sub-family G member 1	0	KOG0065	NA
ASTEI06493-RA	0,0,0	0.371	0.379	0.226	0.629	0.642	0.125	ASTEI06493-RA	*Ae. aegypti*	sulfonylurea receptor/ ABC transporter	0	KOG0054	K05033
ASTEI10920-RA	0,0,0	0.302	0.078	0.313	0.071	0.428	0.016	AGAP006364-PB	*An. gambiae*	ATP-binding cassette, subfamily B (MDR/TAP), member 7	0	KOG0057	K05662
ASTEI06540-RA	0,0,0	-0.066	0.798	-0.124	0.633	-0.158	0.573	AGAP009850-PA	*An. gambiae*	ATP-binding cassette sub-familyG member4	0	KOG0061	NA
ASTEI07390-RA	0,0,0	0.951	1.5e-08	0.118	0.590	-0.700	0.001	AGAP009463-PA	*An. gambiae*	ATP-binding cassette sub-family G member 1	0	KOG0061	NA
ASTEI01604-RA	0,0,1	0.823	0.029	0.172	0.729	1.071	0.004	AGAP007655-PA	*An. gambiae*	Brown protein	0	KOG0061	NA
ASTEI00154-RA	1,0,0	1.057	0.000	0.588	0.000	-0.360	0.004	AGAP002278-PA ADAC008037-PA	*An. gambiae* *An. darlingi*	ATP-binding cassette, subfamily B (MDR/TAP), member 6	0	KOG0056	K05661
ASTEI04427-RA	1,1,1	1.880	0.000	1.844	0.000	1.566	0.000			ABC membrane transporter	0	KOG0061	NA
ASTEI05756-RA	1,1,1	1.680	0.000	3.538	0.000	3.667	0.000	AGAP001333-PA	*An. gambiae*	Scarlet protein	0	KOG0061	NA

Phase I

CYPs

ASTEI00300-RA	-1,-1,-1	-1.837	0.000	-1.125	0.000	-1.329	0.000	AGAP003065-PA	*An. gambiae*	cytochrome P450_CYP11179	0	KOG1667	NA
ASTEI02928-RA	-1,-1,-1	-1.777	0.000	-2.755	0.000	-3.465	0.000	AGAP028019-PA	*An. gambiae*	cytochrome P450_CYP4H18	0	KOG0157	NA
ASTEI02931-RA	-1,-1,-1	-1.769	0.000	-1.471	0.001	-1.999	0.000	AGAP008356-PA	*An. gambiae*	cytochrome P450_CYP4H16	0	KOG0157	NA
ASTEI05843-RA	-1,-1,-1	-1.866	0.001	-2.647	0.000	-4.289	0.000	AGAP011029-PA	*An. gambiae*	cytochrome P450_CYP6AF2	0	KOG0158	K14999
ASTEI02248-RA	-1,-1,0	-1.660	0.000	-1.826	0.000	-0.726	0.002	AGAP005656-PA	*An. gambiae*	cytochrome P450_CYP305A1	0	KOG0156	NA
ASTEI00094-RA	-1,0,-1	-1.085	0.002	-0.882	0.014	-1.134	0.003	AGAP002894-PA	*An. gambiae*	cytochrome P450_CYP6Z4	0	KOG0158	K14999
ASTEI03053-RA	-1,0,-1	-1.789	0.000	-0.229	0.357	-1.789	0.000	AGAP008218-PA	*An. gambiae*	cytochrome P450_CYP6Z3	0	KOG0158	K14999
ASTEI07451-RA	-1,0,0	-1.485	0.004	0.466	0.427	-0.277	0.697	CPIJ012484-PA	*C. quinquefasciatus*	cytochrome P450_CYP6CP1	1E-27	NA	NA
ASTEI06914-RA	-1,0,1	-1.770	0.002	0.383	0.561	1.704	0.006	AAEL004870-PA	*Ae. aegypti*	cytochrome P450_CYP18A1	0	KOG0156	K14985
ASTEI01783-RA	0,-1,-1	-0.332	0.230	-1.164	0.007	-1.498	0.000	AGAP008022-PA	*An. gambiae*	cytochrome P450_CYP12F1	0	KOG0159	K15004
ASTEI11185-RA	0,-1,-1	-0.175	0.427	-1.672	0.000	-2.266	0.000	AGAP010966-PA	*An. gambiae*	cytochrome P450_CYP6AJ1	0	KOG0158	NA
ASTEI11577-RA	0,-1,-1	0.150	0.412	-1.062	0.000	-1.665	0.000	AGAP009363-PA	*An. gambiae*	cytochrome P450_CYP9M1	0	KOG0158	K15003
ASTEI09580-RA	0,-1,0	-1.178	0.064	-1.328	0.042	0.052	0.957	AGAP008682-PA	*An. gambiae*	cytochrome P450_CYP307B1	0	KOG0156	K14939
ASTEI00850-RA	0,0,-1	0.295	0.024	-0.771	0.000	-1.222	0.000	AGAP003343-PA	*An. gambiae*	cytochrome P450_CYP6AG1	0	KOG0158	K14999
ASTEI01072-RA	0,0,-1	0.814	0.000	-0.035	0.860	-1.362	0.000	AGAP000818-PA	*An. gambiae*	cytochrome P450_CYP9K1	0	KOG0158	K15003
ASTEI01187-RA	0,0,-1	0.946	0.257	0.000	1.000	-9.021	0.000	AGAP000877-PA	*An. gambiae*	cytochrome P450_CYP4G17	0	KOG0157	K15001
ASTEI02412-RA	0,0,-1	0.955	0.000	-0.517	0.008	-1.476	0.000	AGAP012296-PA	*An. gambiae*	cytochrome P450_CYP9J5	0	KOG0158	K15003
ASTEI02927-RA	0,0,-1	-0.106	0.481	-0.798	0.000	-1.228	0.000	AGAP008358-PA	*An. gambiae*	cytochrome P450_CYP4	0	KOG0157	NA
ASTEI03059-RA	0,0,-1	-0.499	0.018	-0.840	0.000	-1.467	0.000	AGAP008207-PA	*An. gambiae*	cytochrome P450_CYP6Y2	0	KOG0158	K14999
ASTEI08755-RA	0,0,-1	0.436	0.001	-0.210	0.141	-1.073	0.000	AGAP001864-PA	*An. gambiae*	cytochrome P450_CYP4G16	0	KOG0157	NA
ASTEI08938-RA	0,0,-1	0.948	0.000	-0.236	0.021	-1.083	0.000	AGAP013241-PA	*An. gambiae*	cytochrome P450_CYP4D16	0	KOG0157	NA
ASTEI00453-RA	0,0,0	0.591	0.000	-0.069	0.504	-0.516	0.000	AGAP002866-PA	*An. gambiae*	cytochrome P450_CYP6P4	0	KOG0158	NA
ASTEI02415-RA	0,0,0	0.321	0.010	-0.004	0.980	-0.520	0.000	AGAP012292-PA	*An. gambiae*	cytochrome P450_CYP9J4	0	KOG0158	NA
ASTEI03056-RA	0,0,0	0.244	0.027	-0.135	0.276	-0.840	0.000	AGAP008213-PA	*An. gambiae*	cytochrome P450_CYP6M3	0	KOG0158	NA
ASTEI03060-RA	0,0,0	0.110	0.605	0.781	0.000	0.069	0.764	AGAP008206-PA	*An. gambiae*	cytochrome P450_CYP6N2	0	KOG0158	K14999
ASTEI06230-RA	0,0,0	0.689	0.006	-0.481	0.078	-0.473	0.077	AGAP013490-PA	*An. gambiae*	cytochrome P450_CYP4H24	0	KOG0157	K15001
ASTEI06609-RA	0,0,0	0.802	0.003	0.860	0.002	0.747	0.007	AGAP009240-PA	*An. gambiae*	cytochrome P450_CYP4C35	0	KOG0157	NA
ASTEI07775-RA	0,0,0	0.362	0.016	0.591	0.000	0.353	0.035	AGAP001076-PA	*An. gambiae*	cytochrome P450_CYP4G16	0	KOG0157	K15001
ASTEI09341-RA	0,0,0	-0.001	0.997	-0.647	0.000	-0.386	0.037	AGAP003522-PA	*An. gambiae*	cytochrome P450_CYP329A1	0	KOG0158	NA
ASTEI10310-RA	0,0,0	0.597	0.000	-0.197	0.282	-0.710	0.000	AGAP009363-PA	*An. gambiae*	cytochrome P450_CYP9M1	1E-154	KOG0158	NA
ASTEI10596-RA	0,0,0	-1.342	0.058	0.287	0.747	0.706	0.390	AGAP000194-PA	*An. gambiae*	cytochrome P450_CYP4C25	0	KOG0157	NA
ASTEI10901-RA	0,0,0	0.981	0.000	0.034	0.922	-0.741	0.005	AGAP006047-PA	*An. gambiae*	cytochrome P450_CYP4J9	0	KOG0157	NA
ASTEI00597-RA	0,0,1	0.848	0.017	0.734	0.048	1.411	0.000	AGAP002205-PA	*An. gambiae*	cytochrome P450_CYP325C2	0	KOG0157	NA
ASTEI03057-RA	0,1,0	0.350	0.058	1.229	0.000	0.463	0.013	AAEL009124-PA	*Ae. aegypti*	cytochrome P450_CYP6N12	0	KOG0158	K14999

ASTEI00598-RA	0,1,1	0.491	0.388	1.392	0.006	1.518	0.002	AGAP002211-PA	*An. gambiae*	cytochrome P450_CYP325A1	0	KOG0157	NA
ASTEI08758-RA	1,0,-1	1.023	0.000	-0.487	0.001	-1.645	0.000	AGAP001861-PA	*An. gambiae*	cytochrome P450_CYP4H14	0	KOG0157	NA
ASTEI01400-RA	1,0,0	1.051	0.000	0.186	0.092	-0.184	0.132	AGAP007480-PA	*An. gambiae*	cytochrome P450_CYP6AH1	0	KOG0158	K20474
ASTEI08183-RA	1,0,0	1.301	0.000	0.740	0.001	0.351	0.207	AGAP003608-PA	*An. gambiae*	cytochrome P450_CYP4AA1	0	KOG0157	K15001
ASTEI08939-RA	1,0,0	1.095	0.000	0.708	0.001	0.144	0.595	AGAP002417-PA	*An. gambiae*	cytochrome P450_CYP4AR1	0	KOG0157	NA
ASTEI09029-RA	1,0,0	1.174	0.000	0.505	0.010	0.172	0.480	AGAP010961-PA	*An. gambiae*	cytochrome P450_CYP6AK1	0	KOG0158	K14999
ASTEI08940-RA	1,1,0	2.186	0.000	1.711	0.000	0.552	0.006	AGAP002416-PA	*An. gambiae*	cytochrome P450_CYP4K2	0	KOG0157	NA
ASTEI09473-RA	1,1,0	1.463	0.000	1.054	0.000	0.743	0.003	AGAP010414-PA	*An. gambiae*	cytochrome P450_CYP4C28	0	KOG0157	NA
ASTEI00299-RA	1,1,1	2.599	0.000	2.688	0.000	2.754	0.000	AGAP003066-PA	*An. gambiae*	cytochrome P450_CYP304B1	0	KOG0156	NA
ASTEI06605-RA	1,1,1	1.213	0.002	2.016	0.000	2.459	0.000	AGAP009246-PA	*An. gambiae*	cytochrome P450_CYP4C27	0	KOG0157	NA
AKRs													
ASTEI05819-RA	0,0,0	0.252	0.229	0.505	0.010	0.059	0.828	AAEL004086-PB	*Ae. aegypti*	aldo-ketoreductase	3E-173	KOG1577	K00011
EHs													
ASTEI09322-RA	0,0,0	0.364	0.036	0.236	0.216	-0.346	0.061	AGAP003542-PA	*An. gambiae*	Epoxide hydrolase 2, cytoplasmic, isoform CRA_a		KOG4178	NA
CCEs													
ASTEI06404-RA	-3	-1.294	0.000	-2.007	0.000	-1.818	0.000	AGAP010917-PA	*An. gambiae*	carboxylesterase	0	KOG1516	NA
ASTEI08528-RA	-1,0,-1	-1.770	0.001	-0.519	0.392	-1.314	0.022	AGAP005833-PA	*An. gambiae*	Carboxylesterase juvenile hormone esterase	0	KOG1516	NA
ASTEI00521-RA	0,-1,-1	-0.801	0.000	-1.392	0.000	-1.631	0.000	AGAP013509-PA	*An. gambiae*	Carboxylesterase clade H, member1	0	KOG1516	NA
ASTEI10045-RA	0,-1,-1	0.179	0.380	-1.477	0.000	-2.117	0.000	AGAP006726-PA	*An. gambiae*	carboxylesterase	0	KOG4389	NA
ASTEI04268-RA	0,-1,1	-0.041	0.934	-1.473	0.000	1.352	0.001	AGAP011365-PA	*An. gambiae*	carboxylesterase	0	KOG1516	NA
ASTEI10026-RA	0,0,-1	0.300	0.751	-0.832	0.338	-6.529	0.000	AGAP006700-PA	*An. gambiae*	Carboxylesterase alpha esterase	0	KOG4389	NA
ASTEI02522-RA	0,0,0	0.548	0.129	0.872	0.011	0.726	0.059	AGAP005758-PA	*An. gambiae*	carboxylesterase	0	KOG4389	NA
ASTEI06403-RA	0,0,0	-0.005	0.976	-0.468	0.000	-0.748	0.000	AGAP010911-PA	*An. gambiae*	carboxylesterase	0	KOG1516	NA
ASTEI08970-RA	0,0,0	-1.436	0.061	0.283	0.773	0.344	0.720	AGAP002391-PA	*An. gambiae*	carboxylesterase	0	KOG1516	NA
ASTEI09120-RA	0,0,0	-0.756	0.000	-0.785	0.000	-0.804	0.000	AGAP006228-PA	*An. gambiae*	carboxylesterase	0	KOG1516	NA
ASTEI10044-RA	0,0,0	0.238	0.049	-0.135	0.313	-0.394	0.002	AGAP006723-PA	*An. gambiae*	carboxylesterase	0	KOG4389	NA
ASTEI10069-RA	0,0,0	-0.602	0.000	0.186	0.171	0.813	0.000	AGAP005371-PA	*An. gambiae*	carboxylesterase beta esterase	0	KOG1516	NA
ASTEI10744-RA	0,0,0	0.708	0.005	0.581	0.026	0.168	0.615	AGAP001101-PA	*An. gambiae*	carboxylesterase	0	KOG4389	NA
ASTEI11262-RA	0,0,0	0.707	0.000	0.556	0.003	0.045	0.866	AGAP005757-PA	*An. gambiae*	Carboxylesterase alpha esterase	0	KOG1516	NA
ASTEI11467-RA	0,0,0	0.668	0.077	0.696	0.062	0.895	0.024	AGAP005758-PA	*An. gambiae*	carboxylesterase	3,00E-109	KOG1516	NA
ASTEI00392-RA	1,0,0	1.261	0.001	0.665	0.117	0.487	0.260	AGAP002090-PA	*An. gambiae*	carboxylesterase	0	KOG4389	NA
ASTEI02618-RA	1,0,0	1.257	0.022	0.146	0.847	0.887	0.144	AGAP006956-PA	*An. gambiae*	carboxylesterase	0	KOG4389	NA
ASTEI03121-RA	1,1,0	1.909	0.000	1.051	0.000	0.088	0.616	AGAP001723-PA	*An. gambiae*	Carboxylesterase alpha esterase	0	KOG1516	NA

ASTEI08772-RA	1,1,0	1.631	0.000	1.421	0.000	0.972	0.000	AGAP011507-PA	*An. gambiae*	carboxylesterase	0	KOG1516	NA
ASTEI06752-RA	1,1,1	1.522	0.003	1.837	0.001	1.315	0.012	AGAP010390-PA	*An. gambiae*	carboxylesterase	0	KOG4389	NA
Phase II													
GSTs													
ASTEI10390-RA	-1,-1,-1	-1.044	0.000	-1.705	0.000	-1.317	0.000	AGAP005749-PA	*An. gambiae*	glutathione S-transferase omega class	5.00E-169	KOG0406	K00310
ASTEI11637-RA	-1,-1,-1	-1.188	0.008	-1.929	0.000	-2.933	0.000	AGAP009193-PA	*An. gambiae*	glutathione S-transferase epsilon class	4.00E-73	KOG0867	NA
ASTEI00999-RA	0,-1,-1	-0.683	0.000	-1.831	0.000	-1.475	0.000	AGAP000761-PA	*An. gambiae*	glutathione S-transferase theta class	1.00E-150	KOG0867	K00799
ASTEI03480-RA	0,-1,-1	-0.714	0.006	-1.260	0.000	-1.271	0.000	AGAP000888-PB	*An. gambiae*	glutathione S-transferase theta class	2.00E-136	KOG0867	K00799
ASTEI05223-RA	0,-1,-1	-0.718	0.001	-1.728	0.000	-1.476	0.000	AGAP009195-PA	*An. gambiae*	glutathione S-transferase epsilon class	4.00E-121	KOG0867	K00799
ASTEI05224-RA	0,-1,-1	-0.021	0.916	-1.716	0.000	-1.624	0.000	AGAP009191-PA	*An. gambiae*	glutathione S-transferase epsilon class	4.00E-149	KOG0867	K00799
ASTEI05225-RA	0,-1,-1	-0.739	0.001	-1.037	0.000	-1.116	0.000	AGAP009196-PA	*An. gambiae*	glutathione S-transferase epsilon class	1.00E-150	KOG0867	K00799
ASTEI05226-RA	0,-1,-1	-0.847	0.000	-2.088	0.000	-2.031	0.000	AGAP009197-PA	*An. gambiae*	glutathione S-transferase epsilon class	6.00E-150	KOG0867	K00799
ASTEI07626-RA	0,-1,-1	-0.694	0.002	-1.203	0.000	-1.674	0.000	AGAP004172-PA	*An. gambiae*	glutathione S-transferase delta class	2.00E-147	KOG0867	K00799
ASTEI08702-RA	0,-1,-1	-0.353	0.002	-1.251	0.000	-1.509	0.000	AGAP004379-PA	*An. gambiae*	glutathione S-transferase delta class	1.00E-150	KOG0867	K00799
ASTEI08916-RA	0,-1,-1	-0.594	0.000	-1.276	0.000	-1.366	0.000	AGAP003257-PA	*An. gambiae*	glutathione S-transferase	8.00E-149	KOG0867	K00799
ASTEI10592-RA	0,-1,-1	-0.652	0.002	-1.144	0.000	-1.165	0.000	AGAP000947-PA	*An. gambiae*	glutathione S-transferase	6.00E-146	KOG0867	K00799
ASTEI07618-RA	0,0,0	0.159	0.590	-0.085	0.801	-0.598	0.036	CPIJ002661-PA	*C. quinquefasciatus*	glutathione S-transferase 1-6	0	KOG0867	NA
ASTEI07627-RA	0,0,0	-0.888	0.000	-0.810	0.001	-0.035	0.924	AGAP004173-PA	*An. gambiae*	glutathione S-transferase delta class	2.00E-122	KOG0867	K00799
ASTEI08703-RA	0,0,0	0.123	0.355	-0.389	0.001	-0.462	0.000	AGAP004382-PA	*An. gambiae*	glutathione S-transferase delta class	1.00E-128	KOG0867	NA
ASTEI09484-RA	0,0,0	-0.647	0.000	-0.346	0.029	-0.656	0.000	AGAP010404-PA	*An. gambiae*	glutathione S-transferase	2.00E-146	KOG1695	K04097
ASTEI11664-RA	0,0,0	-0.015	0.957	0.081	0.771	0.247	0.321	AGAP009192-PA	*An. gambiae*	glutathione S-transferase epsilon class	4E-142	KOG0867	K00799
ASTEI00084-RA	0,0,0	0.088	0.841	-0.460	0.266	-0.047	0.919	AGAP002898-PA	*An. gambiae*	glutathione S-transferase zeta class	2E-43	KOG0868	K01800
ASTEI00091-RA	0,0,0	0.468	0.188	-0.183	0.677	0.129	0.777	AGAP002898-PB	*An. gambiae*	glutathione S-transferase zeta class	5E-110	KOG0868	K01800
UGTs													
ASTEI02575-RA	-1,-1,-1	-1.253	0.000	-2.120	0.000	-1.834	0.000	AGAP007029-PA	*An. gambiae*	glucosyl/glucuronosyl transferases	0	KOG1192	K00699
ASTEI00210-RA	0,-1,-1	-0.745	0.000	-1.574	0.000	-1.548	0.000	AGAP002327-PA	*An. gambiae*	glucosyl/glucuronosyl transferases	0	KOG1192	K00699
ASTEI02576-RA	0,-1,-1	-0.142	0.540	-1.089	0.000	-1.246	0.000	AGAP007028-PA	*An. gambiae*	glucosyl/glucuronosyl transferases	0	KOG1192	K00699
ASTEI07685-RA	0,-1,-1	-0.565	0.078	-1.174	0.000	-1.694	0.000	AAEL000687-PA	*Ae. aegypti*	glucosyl/glucuronosyl transferases	0	KOG1192	K00699
ASTEI11135-RA	0,-1,-1	-0.175	0.715	-1.304	0.002	-2.221	0.000	AGAP011564-PA	*An. gambiae*	Glucosyl/glucuronosyl transferases	0	KOG1192	K00699
ASTEI10392-RA	0,-1,0	-0.194	0.257	-1.015	0.000	-0.488	0.004	AGAP005753-PA	*An. gambiae*	glucosyl/glucuronosyl transferases	0	KOG1192	K00699
ASTEI03891-RA	0,0,-1	-0.143	0.790	-0.989	0.049	-1.898	0.000	AGAP011564-PA	*An. gambiae*	Glucosyl/glucuronosyl transferases	0	KOG1192	K00699
ASTEI08260-RA	0,0,-1	-0.124	0.437	-0.921	0.000	-1.514	0.000	AGAP005163-PA	*An. gambiae*	Glucosyl/glucuronosyl transferases	0	KOG1192	K00699

ASTEI08615-RA	0,0,-1	-0.219	0.169	-0.595	0.000	-1.220	0.000	AGAP006775-PA	*An. gambiae*	Glucosyl/glucuronosyl transferases	0	KOG1192	K00699
ASTEI01492-RA	0,0,0	0.198	0.315	0.254	0.197	-0.687	0.000	AGAP007589-PA	*An. gambiae*	Glucosyl/glucuronosyl transferases	0	KOG1192	K00699
ASTEI07688-RA	0,0,0	-0.615	0.000	-0.510	0.003	-0.578	0.002	AGAP007920-PA	*An. gambiae*	Glucuronosyl transferase	0	KOG1192	K00699
ASTEI10974-RA	0,0,0	-0.625	0.034	-0.873	0.003	-0.532	0.101	AGAP002801-PA	*An. gambiae*	Galactosyl galactosyl xylosyl protein 3-beta-glucuronosyltransferase 3	0	KOG1476	K10158
ASTEI00013-RA	1,1,1	1.598	0.000	1.113	0.000	1.614	0.000	AGAP002783-PA	*An. gambiae*	UDP glucuronosyl transferase 5 family, polypeptide A2	0	KOG1192	K00699
Antioxidant enzymes													
ASTEI07113-RA	0,-1,-1	-0.641	0.000	-1.248	0.000	-1.311	0.000	AGAP010517-PA	*An. gambiae*	manganese-iron (Mn-Fe) superoxide dismutase	4E-162	KOG0867	K04564
ASTEI05480-RA	0,0,0	-0.656	0.103	-0.743	0.068	0.438	0.376	AAEL006271-PC	*Ae. aegypti*	copper-zinc (Cu-Zn) superoxide dismutase	0.000008	NA	NA
ASTEI06006-RA	0,0,0	0.135	0.736	0.426	0.230	0.693	0.060	AGAP005234-PA	*An. gambiae*	copper-zinc (Cu-Zn) superoxide dismutase	1E-137	KOG0441	K04565
ASTEI06711-RA	0,0,0	0.157	0.114	-0.257	0.007	-0.486	0.000	AGAP010347-PA	*An. gambiae*	copper-zinc (Cu-Zn) superoxide dismutase	2E-96	KOG0441	K04565
ASTEI10644-RA	0,0,0	0.399	0.000	0.133	0.242	-0.449	0.000	AGAP004904-PA	*An. gambiae*	catalase	0	KOG0047	K03781
ASTEI06040-RA	1,1,1	1.838	0.000	1.927	0.000	1.686	0.000	CPIJ014001-PA	*C. quinquefasciatus*	Superoxide dismutase, Mn	0.000003	NA	NA
HSPs													
ASTEI02621-RA	-1,-1,-1	-3.784	0.000	-1.522	0.000	-1.037	0.000	AAEL014845-PA	*Ae. aegypti*	heat shock protein	3E-99	KOG0019	K04079
ASTEI02622-RA	-1,-1,-1	-4.074	0.000	-1.549	0.000	-1.064	0.000	AGAP006958-PA	*An. gambiae*	Heat shock protein83	0	KOG0020	K04079
ASTEI06698-RA	-1,-1,-1	-2.319	0.000	-1.510	0.000	-1.706	0.000	AGAP010331-PA	*An. gambiae*	heat shock protein 110kDa	0	KOG0103	K09485
ASTEI07645-RA	-1,-1,-1	-1.903	0.000	-1.980	0.000	-1.109	0.000	AGAP004192-PA	*An. gambiae*	heat shock 70kDa protein 5	0	KOG0100	K09490
ASTEI11432-RA	-1,-1,-1	-4.029	0.000	-1.666	0.000	-1.018	0.000	AGAP006958-PA	*An. gambiae*	Heat shock protein83	0	KOG0020	K04079
ASTEI03620-RA	-1,-1,0	-2.068	0.000	-1.586	0.000	-0.815	0.000	AGAP001424-PA	*An. gambiae*	heat shock protein 90kDa beta	0	KOG0020	K09487
ASTEI04786-RA	-1,0,0	-2.969	0.000	0.818	0.016	0.336	0.419	AAEL017973-PA	*Ae. aegypti*	heat shock protein HSP70	0	KOG0101	K03283
ASTEI01441-RA	0,0,0	-0.164	0.042	-0.499	0.000	-0.780	0.000	AGAP013228-PA	*An. gambiae*	Heat shock protein 67B2	7E-74	KOG1530	NA
ASTEI05419-RA	0,0,0	0.317	0.019	0.264	0.057	0.275	0.079	AGAP002076-PA	*An. gambiae*	heat shock 70kDa protein 1/8	0	KOG0101	K03283
ASTEI10589-RA	0,0,0	-0.667	0.000	-0.555	0.000	-0.788	0.000	CPIJ009818-PA	*C. quinquefasciatus*	heat shock protein	7E-65	KOG3591	NA
CPs													
ASTEI02027-RA	-1,0,0	-1.634	0.033	0.064	0.952	0.778	0.374	AGAP009758-PA	*An. gambiae*	Cuticular protein CPLCP11	1E-117	NA	NA
ASTEI02028-RA	-1,0,0	-1.869	0.017	0.393	0.689	0.847	0.332	AGAP009759-PA	*An. gambiae*	Cuticular protein CPLCP12	1E-135	NA	NA
ASTEI02077-RA	-1,0,0	-1.726	0.028	-0.066	0.952	0.620	0.502	AGAP005456-PA	*An. gambiae*	Cuticular protein 15 RR-1 family	3E-85	NA	NA
ASTEI02563-RA	-1,0,0	-1.865	0.017	0.324	0.748	0.367	0.706	AGAP007042-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR62)	4E-100	NA	NA
ASTEI02890-RA	-1,0,0	-1.877	0.015	-1.020	0.223	0.338	0.722	AGAP006095-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR106)	2E-97	NA	NA

ASTEI04631-RA	-1,0,0	-1.589	0.042	-0.131	0.905	0.332	0.724	AGAP006867-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR141)	0	NA	NA
ASTEI04633-RA	-1,0,0	-2.420	0.001	0.075	0.944	0.000	1.000	AGAP006865-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR64)	4E-78	NA	NA
ASTEI04634-RA	-1,0,0	-1.657	0.035	1.335	0.100	0.190	0.846	AGAP006847-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR48)	6E-32	NA	NA
ASTEI04644-RA	-1,0,0	-2.642	0.000	0.029	0.978	0.444	0.642	AGAP006828-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR60)	7E-35	NA	NA
ASTEI04792-RA	-1,0,0	-2.349	0.002	0.545	0.556	0.319	0.744	AGAP004576-PA	*An. gambiae*	Cuticular protein12 in TWDL family	3E-110	NA	NA
ASTEI04959-RA	-1,0,0	-2.339	0.002	0.102	0.927	0.430	0.649	AGAP028002-PA	*An. gambiae*	Cuticular protein (putative) CPLCP7	6E-172	NA	NA
ASTEI04960-RA	-1,0,0	-1.936	0.012	0.131	0.905	0.392	0.678	AGAP028042-PA	*An. gambiae*	Cuticular protien (putative) CPLCP6	6E-78	NA	NA
ASTEI04961-RA	-1,0,0	-2.890	0.000	-0.749	0.375	0.365	0.709	AGAP028018-PA	*An. gambiae*	Cuticular protein (putative) CPLCP24	2E-27	NA	NA
ASTEI05164-RA	-1,0,0	-1.777	0.009	-0.109	0.907	-0.586	0.480	AGAP010717-PA	*An. gambiae*	Cuticular protein RR-2 family (CPR143)	0	NA	NA
ASTEI05444-RA	-1,0,0	-1.811	0.020	0.155	0.887	0.595	0.511	AGAP013269-PA	*An. gambiae*	Cuticular protein10 in TWDL family	3E-159	NA	NA
ASTEI06161-RA	-1,0,0	-2.620	0.000	-0.174	0.873	0.000	1.000	AGAP000345-PA	*An. gambiae*	Cuticular protein RR-2 family (CPR126)	0.00002	NA	NA
ASTEI06228-RA	-1,0,0	-1.354	0.002	-0.407	0.428	0.947	0.060	AGAP000085-PA	*An. gambiae*	Cuticular protein RR-2 family (CPR129)	1E-148	NA	NA
ASTEI06313-RA	-1,0,0	-1.890	0.014	-0.188	0.860	0.321	0.735	AGAP006013-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR33)	6E-97	NA	NA
ASTEI03708-RA	0,0,0	-1.388	0.085	-0.009	0.994	0.539	0.564	AGAP028191-PA	*An. gambiae*	Cuticular protein CPLCW family (CPLCW1)	2E-65	NA	NA
ASTEI03708-RA	0,0,0	-1.388	0.085	-0.009	0.994	0.539	0.564	AGAP028191-PA	*An. gambiae*	Cuticular protein CPLCW family (CPLCW1)	2E-65	NA	NA
ASTEI06558-RA	-1,0,0	-1.645	0.033	-0.012	0.992	1.142	0.169	AGAP009868-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR73)	5E-71	NA	NA
ASTEI06559-RA	-1,0,0	-1.566	0.048	-0.030	0.978	0.279	0.771	AGAP009870-PA	*An. gambiae*	Cuticular protein 151	1E-76	NA	NA
ASTEI06684-RA	-1,0,0	-2.493	0.001	0.090	0.934	0.499	0.595	AGAP028124-PA	*An. gambiae*	Cuticular protein (putative) CPLCP2	2E-19	NA	NA
ASTEI06823-RA	-1,0,0	-2.559	0.001	-0.120	0.913	0.000	1.000	AGAP013465-PA	*An. gambiae*	Cuticular protein (putative) CPLCP1	0.00000006	NA	NA
ASTEI06998-RA	-1,0,0	-2.011	0.003	0.185	0.847	0.112	0.905	AGAP000344-PB	*An. gambiae*	Cuticular protein RR-1 family (CPR127)	8E-12	NA	NA
ASTEI07052-RA	-1,0,0	-1.762	0.023	-0.019	0.986	0.411	0.656	AGAP000352-PA	*An. gambiae*	Cuticular protein TWDL family (TWDL1)	9E-77	NA	NA
ASTEI08986-RA	-1,0,0	-1.644	0.038	-0.160	0.884	-0.016	0.989	AGAP010900-PA	*An. gambiae*	Cuticular protein1 from fifty-one aa family	1E-115	NA	NA
ASTEI09270-RA	-1,0,0	-1.655	0.022	-1.001	0.193	1.503	0.051	AGAP006321-PA	*An. gambiae*	Cuticular protein 71 RR-2 family	1E-97	NA	NA
ASTEI10168-RA	-1,0,0	-2.403	0.002	-0.345	0.730	0.395	0.676	AGAP006868-PB	*An. gambiae*	Cuticular protein RR-1 family (CPR140)	0.00000002	NA	NA
ASTEI10481-RA	-1,0,0	-2.302	0.002	-1.128	0.169	0.355	0.717	AGAP028042-PA	*An. gambiae*	Cuticular protien (putative) CPLCP6	2E-22	NA	NA
ASTEI10495-RA	-1,0,0	-1.935	0.012	-0.669	0.450	0.657	0.473	AGAP006261-PA	*An. gambiae*	Cuticular protein 135 RR-2 family	2E-47	NA	NA
ASTEI10584-RA	-1,0,0	-2.001	0.010	0.047	0.964	0.383	0.686	AGAP000538-PA	*An. gambiae*	Cuticular protein TWDL family (TWDL9)	7E-106	NA	NA

ASTEI11502-RA	-1,0,0	-1.688	0.032	0.170	0.875	0.738	0.410	AGAP006830-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR58)	3E-17	NA	NA
ASTEI11618-RA	-1,0,0	-3.206	0.000	-1.543	0.052	0.537	0.566	AGAP006012-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR32)	1E-33	NA	NA
ASTEI11785-RA	-1,0,0	-3.084	0.000	-1.484	0.064	0.457	0.629	AGAP006012-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR32)	2E-57	NA	NA
ASTEI01032-RA	0,-1,-1	0.778	0.005	-2.585	0.000	-3.175	0.000	AGAP013421-PA	*An. gambiae*	Cuticular protein3 in TWDL family	9E-72	NA	NA
ASTEI03713-RA	0,-1,-1	-0.824	0.052	-1.136	0.006	-1.066	0.010	AGAP008469-PA	*An. gambiae*	Cuticular protein CPLCG family (CPLCG21)	0	NA	NA
ASTEI06564-RA	0,-1,0	0.177	0.713	-1.608	0.000	-0.442	0.384	AGAP009878-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR80)	5E-136	NA	NA
ASTEI03709-RA	0,0,-1	0.989	0.017	-0.644	0.141	-1.562	0.000	AGAP008465-PA	*An. gambiae*	Cuticular protein CPLCG family (CPLCG22)	2E-41	NA	NA
ASTEI05298-RA	0,0,-1	-1.089	0.092	-0.147	0.862	-1.941	0.002	AGAP008444-PA	*An. gambiae*	Cuticular protein CPLCG family (CPLCG1)	4E-50	NA	NA
ASTEI05300-RA	0,0,-1	0.690	0.287	-0.250	0.748	-1.699	0.005	AGAP008446-PA	*An. gambiae*	Cuticular protein CPLCG family (CPLCG3)	5E-56	NA	NA
ASTEI06316-RA	0,0,-1	-0.619	0.230	-0.338	0.550	-1.298	0.012	AGAP006006-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR104)	6E-67	NA	NA
ASTEI06317-RA	0,0,-1	-0.317	0.374	-0.989	0.002	-1.431	0.000	AGAP006005-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR103)	2E-60	NA	NA
ASTEI11099-RA	0,0,-1	0.012	0.988	0.177	0.829	-1.295	0.050	AGAP008460-PA	*An. gambiae*	Cuticular protein CPLCG family (CPLCG16)	8E-50	NA	NA
ASTEI11131-RA	0,0,-1	0.619	0.177	-0.052	0.933	-2.217	0.000	AGAP006011-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR31)	4E-44	NA	NA
ASTEI11350-RA	0,0,-1	0.647	0.268	-0.108	0.886	-1.886	0.000	AGAP008446-PA	*An. gambiae*	Cuticular protein CPLCG family (CPLCG3)	5E-56	NA	NA
ASTEI02026-RA	0,0,0	-1.477	0.056	-0.110	0.917	1.165	0.156	AGAP027993-PA	*An. gambiae*	Cuticular protein (putative) CPLCP10	0	NA	NA
ASTEI02074-RA	0,0,0	-1.528	0.056	-0.054	0.961	0.273	0.780	AGAP005454-PA	*An. gambiae*	Cuticular protein 13 RR-1 family	3E-90	NA	NA
ASTEI02353-RA	0,0,0	-0.714	0.387	0.060	0.954	1.361	0.087	AGAP028013-PA	*An. gambiae*	Cuticular protein (putative) CPLCP25	4E-89	NA	NA
ASTEI02565-RA	0,0,0	-1.485	0.056	-0.760	0.375	0.284	0.758	AGAP007040-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR61)	2E-94	NA	NA
ASTEI02745-RA	0,0,0	0.800	0.021	0.142	0.750	0.458	0.274	AGAP006148-PA	*An. gambiae*	Cuticular protein3 in CPLCA family	4E-54	NA	NA
ASTEI03387-RA	0,0,0	-1.366	0.086	0.061	0.955	0.335	0.721	AGAP003390-PA	*An. gambiae*	Cuticular protein RR-2 family (CPR124)	1E-13	NA	NA
ASTEI03708-RA	0,0,0	-1.388	0.085	-0.009	0.994	0.539	0.564	AGAP028191-PA	*An. gambiae*	Cuticular protein CPLCW family (CPLCW1)	2E-65	NA	NA
ASTEI04635-RA	0,0,0	-1.350	0.090	0.263	0.796	0.232	0.809	AGAP006839-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR67)	5E-153	NA	NA
ASTEI06318-RA	0,0,0	-1.341	0.092	-0.870	0.298	-0.112	0.912	AGAP006002-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR137)	9E-59	NA	NA
ASTEI06561-RA	0,0,0	-0.246	0.585	-0.681	0.089	-0.992	0.020	AGAP009872-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR133)	2E-57	NA	NA
ASTEI06565-RA	0,0,0	0.231	0.397	0.551	0.019	0.998	0.000	AGAP009879-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR81)	8E-74	NA	NA
ASTEI06678-RA	0,0,0	-1.489	0.063	0.281	0.785	0.612	0.507	AGAP012462-PA	*An. gambiae*	Cuticular protein RR-2 family (CPR147)	1E-94	NA	NA
ASTEI06999-RA	0,0,0	-1.413	0.072	0.626	0.475	0.738	0.405	AGAP000345-PA	*An. gambiae*	Cuticular protein RR-2 family	2E-14	NA	NA

										(CPR126)			
ASTEI09421-RA	0,0,0	-0.430	0.628	1.343	0.082	1.031	0.219	AGAP006369-PA	*An. gambiae*	Cuticular protein 144	0	NA	NA
ASTEI02354-RA	0,0,1	-1.055	0.151	-0.447	0.596	1.958	0.007	AGAP028137-PA	*An. gambiae*	Cuticular protein (putative) CPLCP17	8E-94	NA	NA
ASTEI02355-RA	0,0,1	-0.751	0.358	-0.200	0.845	1.685	0.029	AGAP028178-PA	*An. gambiae*	Cuticular protein (putative) CPLCP13	5E-100	NA	NA
ASTEI03181-RA	0,0,1	0.563	0.160	0.978	0.008	2.100	0.000	AGAP002726-PA	*An. gambiae*	Cuticular protein 9 RR-1 family	2E-99	NA	NA
ASTEI05035-RA	0,0,1	-1.198	0.119	0.117	0.911	1.693	0.027	AGAP008817-PA	*An. gambiae*	Cuticular protein (putative) CPLCP3	0	NA	NA
ASTEI08802-RA	0,0,1	0.273	0.690	0.764	0.209	1.420	0.020	AGAP000344-PB	*An. gambiae*	Cuticular protein RR-1 family (CPR127)	2E-121	NA	NA
ASTEI02593-RA	1,0,-1	1.656	0.001	-0.364	0.568	-1.530	0.006	AGAP006931-PA	*An. gambiae*	Cuticular protein 111 RR-3 family	1E-113	NA	NA
ASTEI01256-RA	1,0,0	1.009	0.001	0.322	0.339	-0.228	0.560	AGAP000047-PA	*An. gambiae*	Cuticular protein RR-2 family (CPR130)	1E-150	NA	NA
ASTEI06319-RA	1,0,0	1.193	0.011	0.201	0.744	-0.253	0.674	AGAP005995-PA	*An. gambiae*	Cuticular protein RR-1 family (CPR138)	0	NA	NA
ASTEI08996-RA	1,0,0	1.075	0.000	0.854	0.001	0.592	0.038	AGAP010887-PA	*An. gambiae*	Cuticular protein RR-2 family (CPR113)	2E-149	NA	NA
ASTEI10113-RA	1,0,0	1.217	0.002	0.506	0.245	-0.124	0.825	AGAP006497-PA	*An. gambiae*	Cuticular protein 134	0	NA	NA
Ahr													
ASTEI09831-RA	0,0,-1	-0.390	0.363	-0.041	0.940	-1.760	0.000	AAEL011825-PC	*Ae. aegypti*	Aryl hydrocarbon receptor	0	KOG3560	NA
ASTEI02023-RA	0,0,0	-0.128	0.473	0.238	0.154	0.277	0.085	AGAP009748-PA	*An. gambiae*	aryl hydrocarbon receptor nuclear translocator	0	KOG3561	K09097
ASTEI04432-RA	0,1,0	0.794	0.000	1.065	0.000	0.811	0.000	AGAP000725-PA	*An. gambiae*	Aryl hydrocarbon receptor interacting protein	0	KOG0545	K17767
NRs													
ASTEI02952-RA	-1,0,0	-1.382	0.037	0.261	0.756	-0.115	0.895	AGAP008334-PA	*An. gambiae*	nuclear receptor subfamily 4 group A member 2	0	KOG4217	K08558
ASTEI08516-RA	0,-1,0	0.265	0.699	-2.382	0.000	-0.873	0.255	AGAP006571-PA	*An. gambiae*	nuclear receptor subfamily 1 group D member 3	7E-56	NA	NA
ASTEI02478-RA	0,0,0	-0.110	0.752	-0.259	0.431	0.025	0.955	AAEL007397-PC	*Ae. aegypti*	Ecdysone-induced protein 75B isoform A Nuclear receptor	0	KOG4216	K08701
ASTEI02479-RA	0,0,0	-0.752	0.228	-0.359	0.613	-0.181	0.827	AGAP012223-PA	*An. gambiae*	nuclear receptor subfamily 1 group D member 3	1E-52	KOG4846	NA
ASTEI07992-RA	0,0,0	-0.324	0.523	-0.282	0.589	-0.594	0.214	AGAP002544-PA	*An. gambiae*	nuclear receptor subfamily 2 group F member 3	6E-97	NA	NA
ASTEI07993-RA	0,0,0	-0.537	0.350	-0.125	0.858	-0.300	0.644	AGAP002544-PB	*An. gambiae*	nuclear receptor subfamily 2 group F member 3	0	KOG4215	NA
ASTEI07994-RA	0,0,0	-0.137	0.847	0.112	0.897	0.099	0.898	AAEL002765-PA	*Ae. aegypti*	Seven up nuclear receptor	4E-59	KOG4215	NA
ASTEI08329-RA	0,0,0	-0.356	0.585	0.594	0.355	0.579	0.407	AGAP010438-PA	*An. gambiae*	nuclear receptor subfamily 0 group A	3E-38	KOG4216	K08706

ASTEI08836-RA	0,0,0	0.611	0.355	0.466	0.518	0.342	0.649	AGAP000981-PA	*An. gambiae*	Nuclear receptor interaction protein	3E-53	KOG1310	K11795
ASTEI10441-RA	0,0,0	-1.190	0.116	-0.008	0.994	-0.252	0.796	AGAP004693-PA	*An. gambiae*	nuclear receptor subfamily 6 group A	4E-53	NA	NA
ASTEI10442-RA	0,0,0	-1.051	0.197	-0.148	0.891	-0.009	0.993	AGAP004693-PA	*An. gambiae*	nuclear receptor subfamily 6 group A	0	KOG4218	K09185
ASTEI06629-RA	0,0,0	0.211	0.239	-0.010	0.966	0.435	0.009	AGAP008382-PA	*An. gambiae*	nuclear receptor subfamily 2 group C	0	KOG4215	NA
ASTEI01071-RA	0,0,0	1.029	0.122	1.141	0.086	0.350	0.674	AGAP000819-PA	*An. gambiae*	nuclear receptor subfamily 2 group E member (Tailless)	0	KOG4215	K08545
ASTEI07347-RA	0,0,0	-0.343	0.105	-0.577	0.004	-0.435	0.054	AGAP009400-PA	*An. gambiae*	nuclear receptor subfamily 5 group A member 3	0	KOG4218	K08705
ASTEI10443-RA	0,0,0	-1.479	0.057	0.175	0.868	-0.099	0.923	AGAP004693-PA	*An. gambiae*	nuclear receptor subfamily 6 group A	8E-39	NA	NA
ASTEI03690-RA	0,0,0	0.542	0.001	-0.003	0.991	-0.183	0.325	AGAP004224-PA	*An. gambiae*	nuclear receptor subfamily 1 group I	0	KOG4215	K14035
ASTEI08331-RA	0,0,0	0.561	0.098	-0.526	0.152	0.146	0.763	AGAP010438-PA	*An. gambiae*	nuclear receptor subfamily 0 group A	0	KOG4216	NA
ASTEI04688-RA	0,0,0	0.364	0.005	0.243	0.078	0.050	0.775	AGAP009664-PA	*An. gambiae*	nuclearreceptorcoactivator2	0	NA	K11255
ASTEI01760-RA	0,0,0	0.773	0.000	0.818	0.000	0.385	0.022	AGAP007996-PB	*An. gambiae*	Nuclear receptor-binding protein	0	KOG1266	K08875
MAPK													
ASTEI01876-RA	0,0,0	0.161	0.799	-0.649	0.272	-0.497	0.436	AGAP008129-PA	*An. gambiae*	mitogen-activated protein kinase organizer 1	0	KOG0316	K13124
ASTEI03404-RA	0,0,0	-0.285	0.252	-0.211	0.435	0.071	0.821	AGAP003365-PA	*An. gambiae*	mitogen-activated protein kinase kinase 4	0	KOG1006	K04430
ASTEI10746-RA	0,0,0	0.096	0.726	-0.238	0.373	0.015	0.965	AGAP001103-PA	*An. gambiae*	mitogen-activated protein kinase kinase 1	0	KOG0581	K04368
ASTEI08749-RA	0,0,0	0.189	0.174	0.272	0.042	0.256	0.068	AGAP001867-PA	*An. gambiae*	mitogen-activated protein kinase kinase 7	0	KOG0983	K04431
ASTEI10965-RA	0,0,0	0.241	0.240	0.372	0.063	0.528	0.007	AGAP000310-PA	*An. gambiae*	mitogen-activated protein kinase kinase 3	0	KOG0984	K04432
ASTEI03516-RA	0,0,0	0.429	0.056	0.282	0.270	0.118	0.675	AGAP000997-PA	*An. gambiae*	mitogen-activated protein kinase kinase 1 interacting protein 1	2E-86	NA	K04370
ASTEI00503-RA	0,0,0	0.489	0.097	0.462	0.128	0.693	0.021	AGAP002953-PA	*An. gambiae*	Mitogen-activated protein kinase kinase kinase 7-interacting protein 1	0	NA	K04403
ASTEI02510-RA	0,0,0	0.494	0.000	0.386	0.006	0.063	0.717	AGAP010837-PA	*An. gambiae*	mitogen-activated protein kinase kinase kinase kinase 5	0	KOG0576	K08833
ASTEI04318-RA	0,0,0	0.550	0.000	0.231	0.059	0.127	0.370	CPIJ001610-PA	*C. quinquefasciatus*	mitogen-activated protein kinase kinasekinase 4	0	KOG4645	K04428

ASTEI05089-RA	0,0,0	0.336	0.088	0.330	0.093	0.616	0.001	AGAP011890-PA	*An. gambiae*	mitogen-activated protein kinase-activated protein kinase 2	8E-166	KOG0604	K04443
ASTEI10136-RA	0,0,0	0.618	0.000	0.731	0.000	0.387	0.002	AGAP006461-PA	*An. gambiae*	mitogen-activated protein kinase kinase kinase 13	0	KOG4721	K04422
ASTEI06997-RA	0,1,1	0.926	0.000	1.942	0.000	1.754	0.000	AGAP000747-PB	*An. gambiae*	mitogen-activated protein kinase kinase kinase 5	0	KOG4279	K04426
Nrf2													
ASTEI10187-RA	0,0,0	0.036	0.900	0.293	0.242	0.010	0.978	AGAP005300-PA	*An. gambiae*	Nuclear factor erythroid 2, invertebrate	0	NA	NA
ASTEI10188-RA	0,0,0	0.688	0.000	0.558	0.000	0.164	0.146	AGAP005300-PA	*An. gambiae*	Nuclear factor erythroid 2, invertebrate	0	KOG3863	K09041
ASTEI05762-RA	0,1,0	0.364	0.102	2.297	0.000	0.564	0.014	AGAP001324-PA	*An. gambiae*	Erythroiddifferentiation-relatedfactor1	0	NA	NA
HSF													
ASTEI05581-RA	0,0,0	0.101	0.528	0.306	0.030	0.376	0.011	AAEL010319-PA	*Ae. aegypti*	heat shock transcription factor (hsf)	2E-107	KOG0627	NA
Others xenobiotics defense-related genes													
ASTEI00237-RA	0,0,0	-0.268	0.488	-0.365	0.342	-0.308	0.457	AAEL008369-PA	*Ae. aegypti*	acyl phosphatase, putative	2E-56	KOG3360	K01512
ASTEI00250-RA	0,0,0	0.022	0.907	-0.540	0.000	-0.345	0.030	AGAP003124-PA	*An. gambiae*	dihydropyrimidinase	0	KOG2584	K01464
ASTEI00806-RA	0,0,0	0.253	0.252	0.718	0.000	0.689	0.001	AGAP003874-PC	*An. gambiae*	uridine kinase	0	KOG4203	K00876
ASTEI01275-RA	0,0,0	-0.605	0.459	0.207	0.836	0.485	0.564	AGAP007300-PA	*An. gambiae*	alkaline phosphatase	0	KOG4126	K01077
ASTEI01329-RA	0,0,0	0.355	0.560	0.288	0.683	0.441	0.514	AGAP007374-PA	*An. gambiae*	glucuronosyltransferase	0	KOG1192	K00699
ASTEI01662-RA	-1,-1,-1	-1.713	0.001	-2.430	0.000	-1.516	0.015	AGAP007793-PA	*An. gambiae*	Regucalcin protein	0	KOG4499	K00699
ASTEI01671-RA	0,-1,-1	-0.814	0.000	-1.716	0.000	-1.360	0.000	AGAP007784-PA	*An. gambiae*	enoyl-CoA hydratase / long-chain 3-hydroxyacyl-CoA dehydrogenase	0	KOG1683	K07515
ASTEI02231-RA	1,1,1	1.569	0.001	1.113	0.018	1.232	0.005	AGAP005637-PA	*An. gambiae*	aldehyde oxidase	0	KOG0430	K00106
ASTEI02232-RA	1,1,1	2.061	0.000	2.147	0.000	1.789	0.000	AGAP005638-PA	*An. gambiae*	aldehyde oxidase	0	KOG0430	K00106
ASTEI02833-RA	1,1,0	1.675	0.000	1.054	0.016	0.380	0.501	AGAP005865-PA	*An. gambiae*	fumarylacetoacetase	0	KOG2843	K01555
ASTEI02975-RA	0,0,-1	-0.670	0.046	0.087	0.851	-1.314	0.000	AGAP008311-PA	*An. gambiae*	acylphosphatase	7E-71	KOG3360	K01512
ASTEI03083-RA	0,-1,0	0.945	0.057	-1.457	0.003	0.168	0.810	AGAP001684-PA	*An. gambiae*	alkaline phosphatase	0	KOG4126	K01078
ASTEI03532-RA	0,-1,0	0.706	0.000	-1.215	0.000	-0.447	0.036	AGAP001021-PA	*An. gambiae*	dihydropyrimidine dehydrogenase (NADP+)	0	KOG1799	K00207

ASTEI04027-RA	0,-1,0	-0.756	0.074	-1.075	0.009	-0.521	0.270	AGAP009945-PA	*An. gambiae*	dihydrodiol dehydrogenase / D-xylose 1-dehydrogenase (NADP)	0	KOG2741	K00078
ASTEI04028-RA	0,0,-1	-0.030	0.878	-0.967	0.000	-1.508	0.000	AGAP009944-PA	*An. gambiae*	aldehyde dehydrogenase (NAD+)	0	KOG2450	K00128
ASTEI04208-RA	1,1,0	1.987	0.000	1.027	0.000	-0.488	0.004	AGAP011302-PA	*An. gambiae*	alkaline phosphatase	0	KOG4126	K01079
ASTEI04209-RA	1,1,0	1.261	0.000	1.501	0.000	0.246	0.196	AGAP011305-PB	*An. gambiae*	alkaline phosphatase	0	KOG4126	K01080
ASTEI04235-RA	0,-1,-1	-0.935	0.000	-1.837	0.000	-1.322	0.000	CPIJ019232-PA	*C. quinquefasciatus*	acetyl-CoA acetyltransferase, mitochondrial	0	KOG1390	K00626
ASTEI04371-RA	0,0,0	0.608	0.001	0.168	0.438	0.182	0.453	AGAP003578-PA	*An. gambiae*	aldehyde dehydrogenase (NAD+)	0	KOG2450	K00128
ASTEI04736-RA	1,0,0	1.619	0.000	0.763	0.087	0.342	0.540	AGAP009609-PA	*An. gambiae*	homogentisate 1,2-dioxygenase	0	KOG1417	K00451
ASTEI04740-RA	1,0,0	1.203	0.000	0.779	0.000	0.651	0.001	AGAP008501-PA	*An. gambiae*	glutaryl-CoA dehydrogenase	0	KOG0138	K00252
ASTEI04789-RA	0,0,-1	0.663	0.012	-0.631	0.028	-2.119	0.000	AGAP012634-PA	*An. gambiae*	Alkaline phosphatase	0	KOG4126	K01081
ASTEI05071-RA	0,0,0	0.560	0.000	-0.380	0.000	-0.815	0.000	AGAP011859-PA	*An. gambiae*	beta-glucuronidase	0	KOG2024	K01195
ASTEI05340-RA	0,0,0	-0.708	0.251	0.091	0.910	1.213	0.052	AGAP010596-PA	*An. gambiae*	alkaline phosphatase	0	KOG4126	K01082
ASTEI05639-RA	-1,0,0	-1.227	0.000	-0.616	0.001	-0.411	0.046	AGAP011133-PA	*An. gambiae*	Inosine-5-monophosphate dehydrogenase	0	KOG2550	K00088
ASTEI05718-RA	-1,0,0	-1.130	0.000	-0.240	0.342	-0.033	0.917	AGAP001501-PA	*An. gambiae*	Regucalcin protein	0	KOG4499	K01054
ASTEI05767-RA	-1,-1,-1	-1.183	0.000	-1.681	0.000	-1.067	0.000	AGAP001318-PA	*An. gambiae*	acetyl-CoA C-acetyltransferase	0	KOG1390	K00626
ASTEI06032-RA	0,0,0	0.284	0.088	0.487	0.003	0.301	0.095	AGAP005209-PA	*An. gambiae*	uridine kinase	0	KOG4203	K00876
ASTEI06079-RA	0,0,0	0.383	0.000	-0.269	0.021	-0.495	0.000	CPIJ006479-PA	*C. quinquefasciatus*	3-hydroxyacyl-coa dehyrogenase	0	KOG2304	K00022
ASTEI06183-RA	-1,0,0	-1.833	0.017	0.085	0.937	1.352	0.096	AGAP009137-PA	*An. gambiae*	Ecdysteroid UDP-glucosyltransferase	0	KOG1192	K00699
ASTEI06614-RA	-1,-1,-1	-1.399	0.000	-1.578	0.000	-1.297	0.000	CPIJ000349-PA	*C. quinquefasciatus*	UDP-glucuronosyltransferase 1-1	0	KOG1192	K00699
ASTEI06637-RA	0,-1,0	-0.849	0.000	-1.056	0.000	-0.678	0.003	AGAP008374-PA	*An. gambiae*	inosine triphosphate pyrophosphatase	1E-126	KOG3222	K01519
ASTEI06805-RA	1,1,1	1.409	0.000	1.866	0.000	1.190	0.000	CPIJ002095-PA	*C. quinquefasciatus*	alkaline phosphatase	9E-24	KOG4126	K01083
ASTEI02975-RA	0,0,-1	-0.670	0.046	0.087	0.851	-1.314	0.000	AGAP008311-PA	*An. gambiae*	acylphosphatase	7E-71	KOG0022	K01512
ASTEI03083-RA	0,-1,0	0.945	0.057	-1.457	0.003	0.168	0.810	AGAP001684-PA	*An. gambiae*	alkaline phosphatase	0	KOG2450	K01078
ASTEI03532-RA	0,-1,0	0.706	0.000	-1.215	0.000	-0.447	0.036	AGAP001021-PA	*An. gambiae*	dihydropyrimidine dehydrogenase (NADP+)	0	KOG1377	K00207
ASTEI04027-RA	0,-1,0	-0.756	0.074	-1.075	0.009	-0.521	0.270	AGAP009945-PA	*An. gambiae*	dihydrodiol dehydrogenase / D-xylose 1-dehydrogenase (NADP)	0	KOG0430	K00078
ASTEI04028-RA	0,0,-1	-0.030	0.878	-0.967	0.000	-1.508	0.000	AGAP009944-PA	*An. gambiae*	aldehyde dehydrogenase (NAD+)	0	KOG0430	K00128
ASTEI04208-RA	1,1,0	1.987	0.000	1.027	0.000	-0.488	0.004	AGAP011302-PA	*An. gambiae*	alkaline phosphatase	0	KOG0430	K01079
ASTEI04209-RA	1,1,0	1.261	0.000	1.501	0.000	0.246	0.196	AGAP011305-PB	*An. gambiae*	alkaline phosphatase	0	KOG0808	K01080

ASTEI04235-RA	0,-1,-1	-0.935	0.000	-1.837	0.000	-1.322	0.000	CPIJ019232-PA	*C. quinquefasciatus*	acetyl-CoA acetyltransferase, mitochondrial	0	KOG1622	K00626
ASTEI04371-RA	0,0,0	0.608	0.001	0.168	0.438	0.182	0.453	AGAP003578-PA	*An. gambiae*	aldehyde dehydrogenase (NAD+)	0	KOG0430	K00128
ASTEI04736-RA	1,0,0	1.619	0.000	0.763	0.087	0.342	0.540	AGAP009609-PA	*An. gambiae*	homogentisate 1,2-dioxygenase	0	KOG0430	K00451
ASTEI07133-RA	0,0,0	-0.413	0.001	-0.515	0.000	-0.243	0.108	AGAP010499-PA	NA	S-(hydroxymethyl)glutathione dehydrogenase /Alcohol dehydrogenase (Kegg orthology)	NA	KOG1680	K00121
ASTEI07414-RA	0,0,0	-0.512	0.000	-0.681	0.000	-0.720	0.000	AGAP003652-PB	*An. gambiae*	aldehyde dehydrogenase (NAD+)	0	KOG4126	K00128
ASTEI07629-RA	-1,0,0	-1.580	0.000	-0.943	0.000	-0.737	0.004	AGAP004175-PA	*An. gambiae*	uridine monophosphate synthetase	0	KOG3728	K13421
ASTEI07691-RA	0,0,0	0.475	0.003	0.724	0.000	0.272	0.148	AGAP007918-PA	*An. gambiae*	xanthine dehydrogenase/oxidase	0	KOG1192	K00106
ASTEI07692-RA	0,0,0	0.474	0.001	-0.012	0.953	-0.320	0.044	AGAP007918-PA	*An. gambiae*	xanthine dehydrogenase/oxidase	0	KOG0430	K00106
ASTEI07693-RA	0,0,0	0.475	0.043	0.563	0.022	0.193	0.517	AGAP007918-PA	*An. gambiae*	xanthine dehydrogenase/oxidase	6E-169	KOG2843	K00106
ASTEI07874-RA	1,1,0	1.773	0.000	1.960	0.000	0.980	0.000	AGAP010229-PA	*An. gambiae*	beta-ureidopropionase	0	KOG3360	K01431
ASTEI08316-RA	0,0,0	-0.375	0.002	-0.281	0.025	-0.071	0.651	AGAP010139-PD	*An. gambiae*	GMP synthase (glutamine-hydrolysing)	0	KOG4126	K01951
ASTEI09117-RA	1,1,0	1.548	0.000	1.020	0.002	0.730	0.052	AGAP006221-PA	*An. gambiae*	aldehyde oxidase	0	KOG1799	K00106
ASTEI09118-RA	0,0,0	-0.050	0.773	-0.358	0.011	0.289	0.073	AGAP006226-PA	*An. gambiae*	xanthine dehydrogenase/oxidase	0	KOG2741	K00106
ASTEI09239-RA	0,-1,-1	-0.681	0.000	-1.680	0.000	-1.306	0.000	AGAP011833-PA	*An. gambiae*	enoyl-CoA hydratase	0	KOG2450	K07511
ASTEI09453-RA	-1,-1,-1	-2.458	0.000	-3.334	0.000	-1.339	0.002	AGAP006400-PA	*An. gambiae*	Alkaline phosphatase 2	0	KOG4126	K01084
ASTEI10601-RA	0,-1,0	-0.659	0.000	-1.106	0.000	-0.484	0.017	AGAP000188-PA	*An. gambiae*	uridine phosphorylase	0	KOG4126	K00757
ASTEI11082-RA	-1,-1,-1	-1.204	0.000	-1.386	0.000	-1.600	0.000	CPIJ000351-PA	*C. quinquefasciatus*	UDP-glucuronosyltransferase 2B28	0	KOG1390	K00699

Table S5. Primers used in RT-qPCR validation. List of the primer pairs used to amplify the target genes selected to validate the transcriptomic results.

An. stephensi ID	Gene target	Forward primer (5'-3')	Reverse primer (5'-3')	PCR product size (base pairs)	Source
ASTEI02233-RA	ABC-B2	TATCAAGTTCACGGATGTAGAGT	TATCCACCTTGCCACTGTC	185	[33]
ASTEI05756-RA	ABC-G4	ATGAGCCCATTCGTCCTG	AGCGTGGAGAAGAAGCAG	158	[33]
ASTEI08940-RA	CYP4K2	GCTGAGTTCCATCCTGTAC	TTTCCTCCTCCTCCTGCT	210	This study
ASTEI05843-RA	CYP6AF2	TTTTTACCGTTTGGGGAGGG	CCTGTGACTGTAGCGTAAC	169	This study
ASTEI06404-RA	CARB1	AGGGATCAAAGATGCGACA	CTCCATCAGATATTCGGC	219	This study
ASTEI06752-RA	CARB2	GAAACCTTGGTCTGCTCG	TGAAAAGATTGTGCCCGACATA	199	This study
ASTEI05226-RA	GST2	GAAAGATGACGCCCTCTAC	TCTGTCCCCAGTAAAACACC	135	This study
ASTEI01032-RA	CuPro3	GCCATCTCTAACTCGTTC	CCGAAACCACCATCGAAT	154	This study
	Rps7	AGCAGCAGCAGCACTTGATTTG	TAAACGGCTTTCTGCGTCACCC	90	[33]

Figure S1. Maximum likelyhood phylogenetic tree of ABC transporter family. Colors show the ABC transporter sub-families: ABCBA (green), ABCB (blue); ABCC (magenta); ABCD (red); ABCE (violet); ABCF (yellow); ABCG (brown). The code at the end of the branches represents the ID of each ABC transporter gene detected in *Anopheles stephensi*. The numbers on the branch node indicate the bootstrap values.

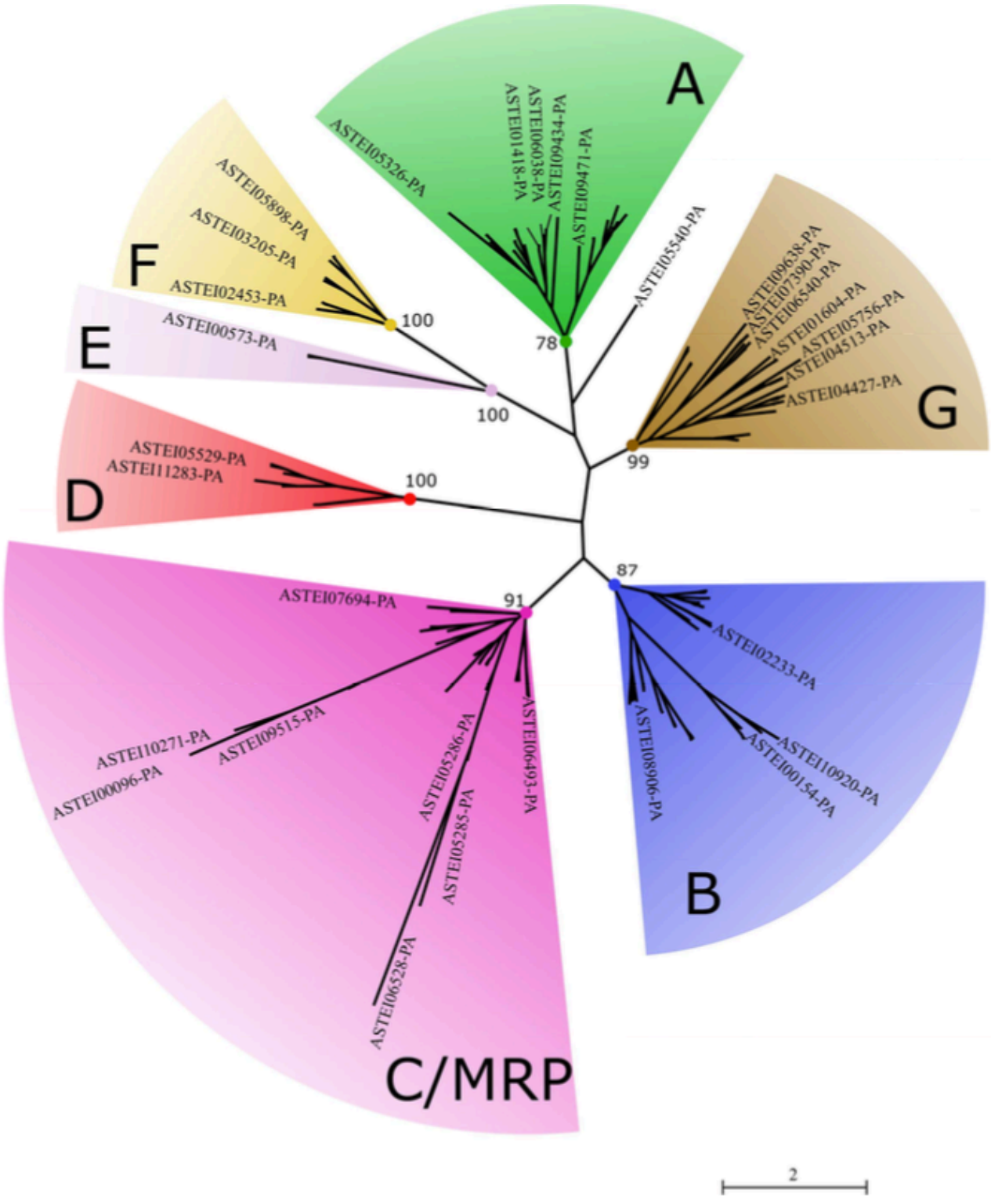


Figure S2. Venn diagrams. Proportions of *Anopheles stephensi* up- and down-regulated genes encoding for Phase I, Phase II, Phase 0/III enzymes and Cuticular Proteins (CPs) after six, 24 and 48 hours.

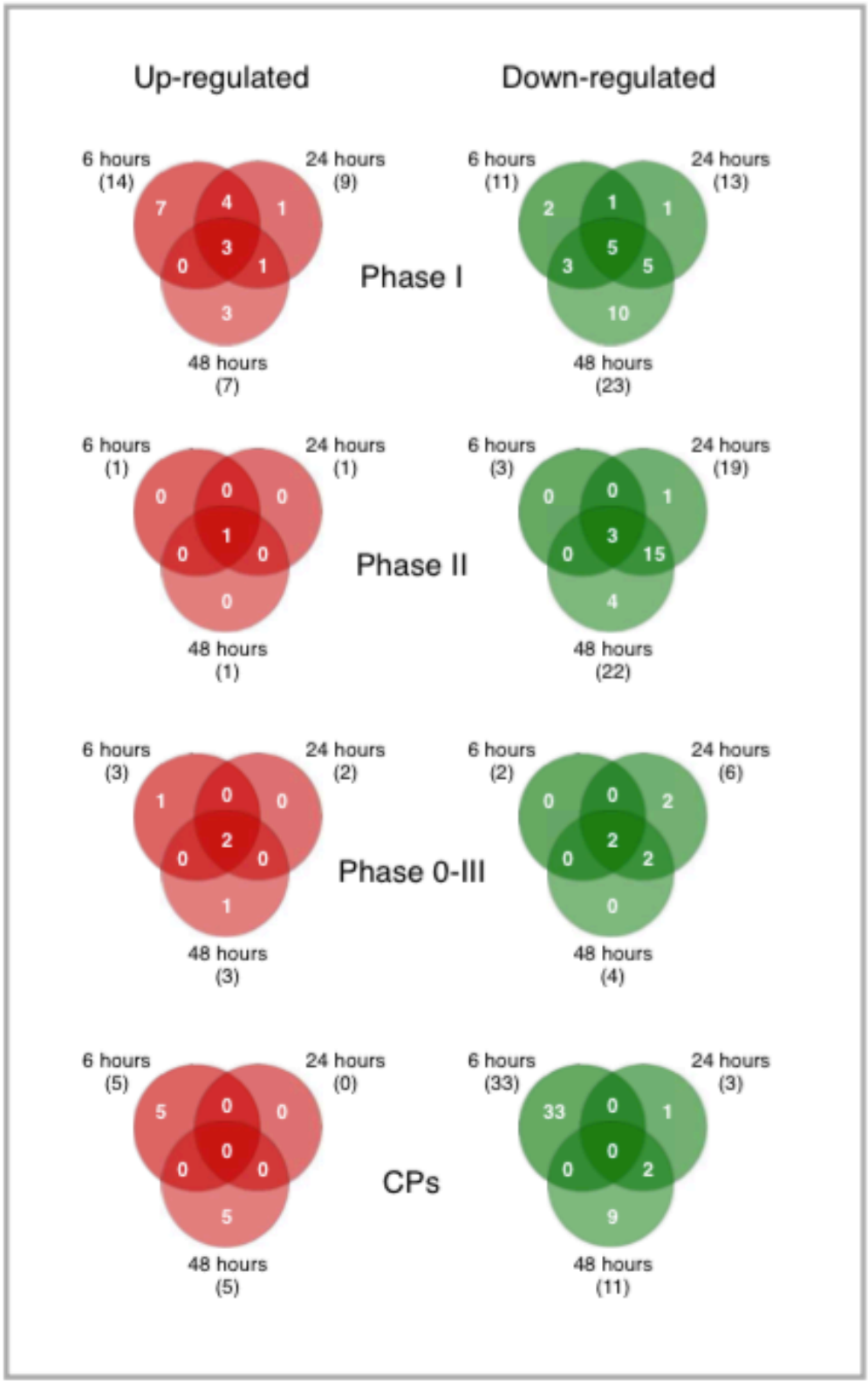


Figure S3. Cluster analysis of insecticide defence-related genes differentially expressed at six, 24 and 48h. Hierarchical clustering analysis based on log fold-change. Genes not differentially expressed at any time point were excluded from the analysis. For each gene, the ID is also indicated. Genes included in the analysis were: ABC transporters, CYPs, CCEs, AKRs, EH, GSTs, UGTs, antioxidant enzymes, HSPs, CPs and KEGG orthologs belonging to the “xenobiotics biodegradation and metabolism” pathways.

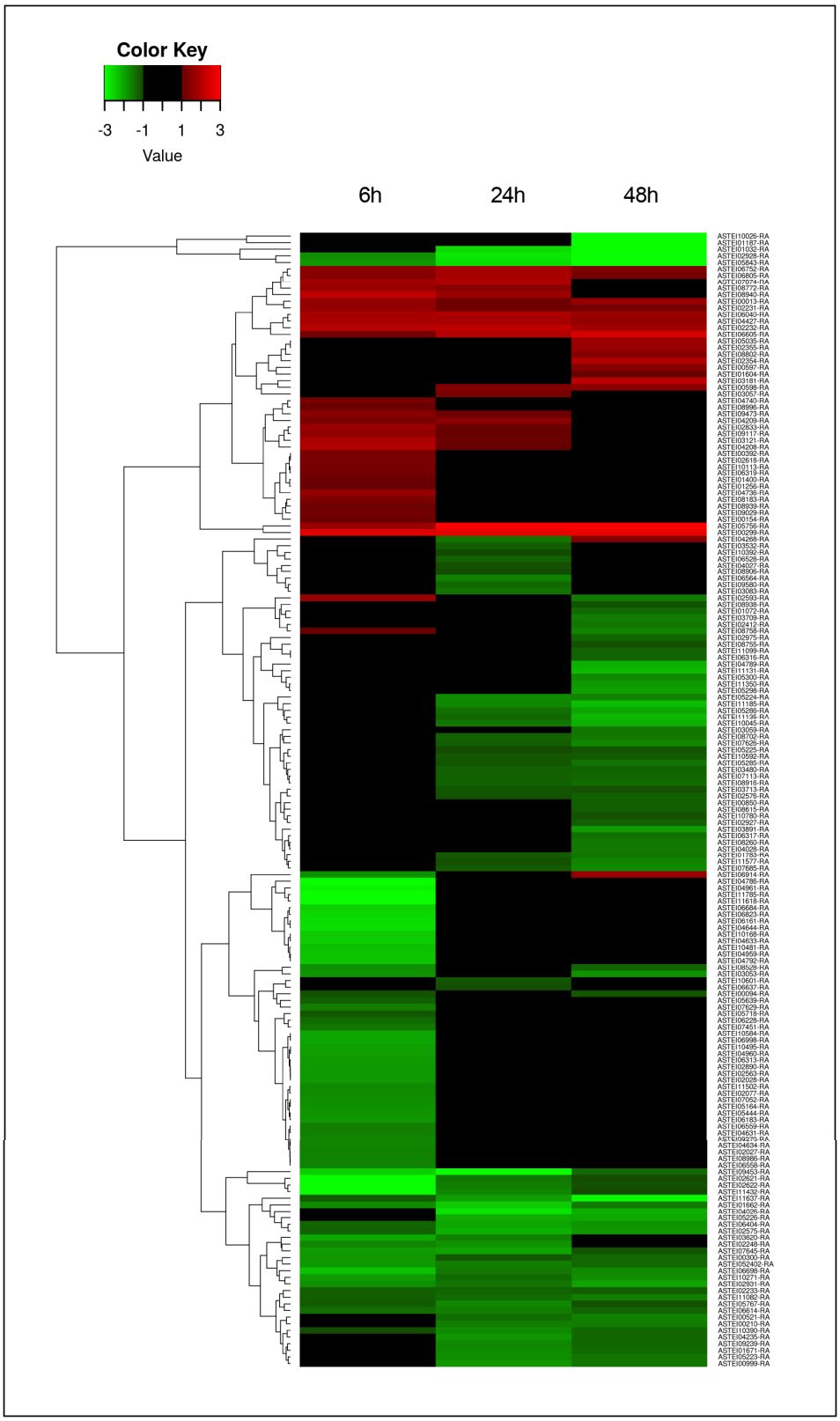


Figure S4. Cluster analysis of Cuticular Proteins. Clustering of genes encoding for Cuticular Proteins detected as differentially expressed at six, 24 and 48h of permethrin exposure. The Roman letters above the clusters refers to the main groups and several sub-groups as indicated in the Results section.

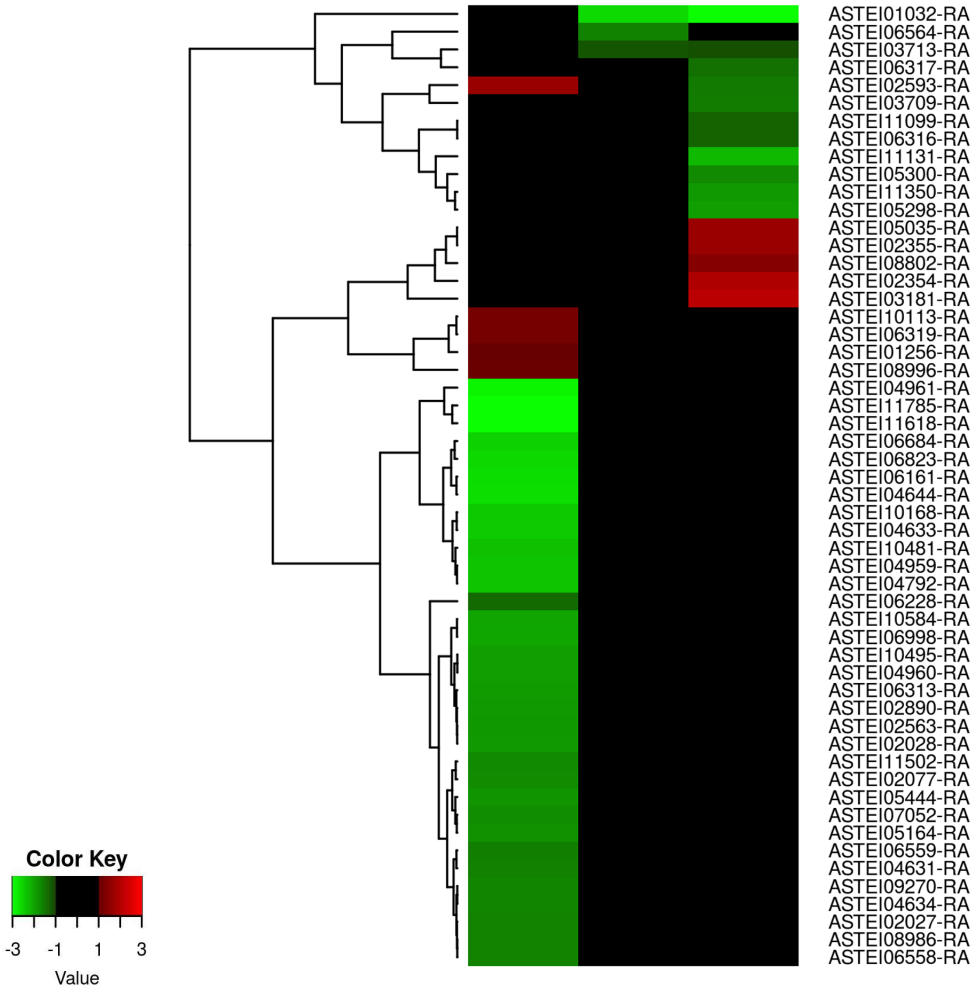
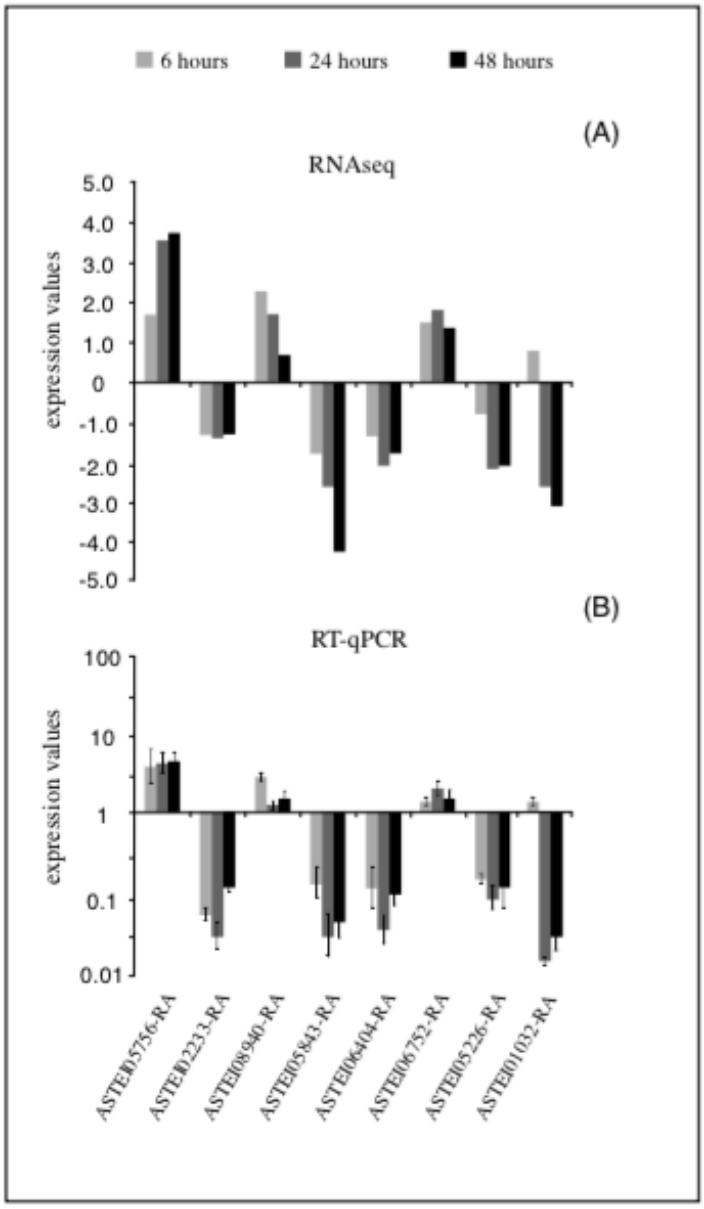


Figure S5. Validation of RNA-seq data by RT-qPCR. (A) RNA-Seq data. The expression value is expressed as log2FC. A gene was considered differentially expressed compared to untreated larvae if its adjusted p-value was less than 0.05 and its absolute log2 fold change was greater than 1. (B) RT-qPCR results. The expression level in untreated larvae was considered the basal level, which was set to 1. The internal reference gene rps7 for *Anopheles stephensi* was used to normalize expression levels. The values are expressed as means of three values and the bars show the standard deviations.



PAPER 2:

Supplemental matherial

Table S1. Mortality of male and female *Anopheles stephensi* adults exposed to permethrin. The results obtained for permethrin treatment by the Student's *t*-test were also shown. df = degree of freedom; SD = standard error of difference.

Time-points	Permethrin mortality rate (%)						Control Mortality rate (%)	
	♂	♀	Student's *t*-test				♂	♀
			t-statistics	*df*	*SD*	*P*-value		
1 h	70 (±11)	40 (±8)	10.398	6	2.844	0.00006	4 (±1)	2 (±1)
24 h	82 (±7)	55 (±12)	4.597	6	5.935	0.0037	5 (±3)	3 (±2)

Table S2. Relative expression of Anopheles stephensi ABC transporter genes measured by quantitative RT-PCR after permethrin treatment. The expression level in non-treated adults was considered to be the basal level (equal 1). The internal reference gene rps7 for An. stephensi was used to normalize expression levels. The values are expressed as fold change mean ± standard deviation

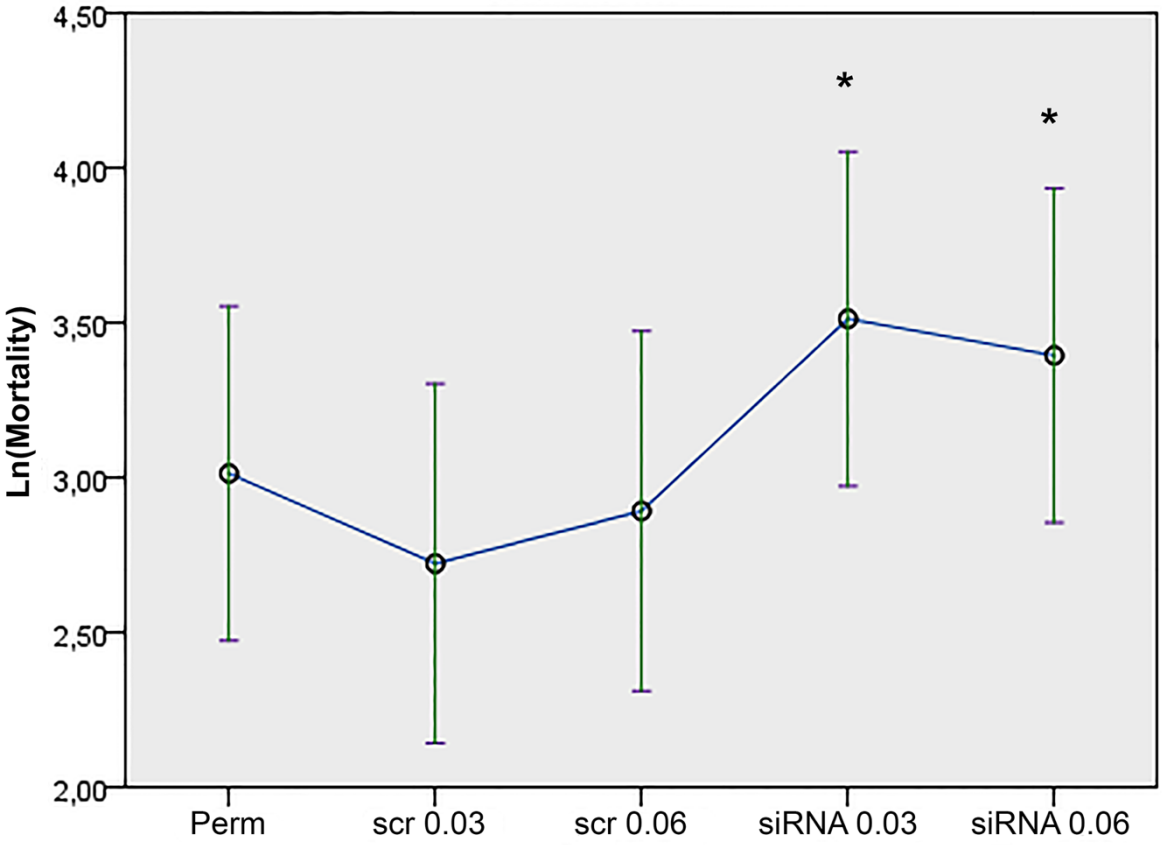
Exposure time	ABCG4	ABCB2	ABCB6	ABCB3	*A*BCB4	ABCC11
Females						
1hour	4.93±0.08	8.51±1.17	8.75±1.69	0.98±0.20	0.98±0.19	1.28±0.07
24 hours	3.38±0.18	9.15±0.15	6.45±0.15	1.13±0.14	1.62±0.04	1.64±0.12
Males						
1hour	6.27±0.09	3.2±0.17	2.7±0.08	0.94±0.06	0.66±0.18	0.42±0.48
24 hours	4.4±0.08	8.99±0.15	7.98±0.09	1.54±0.14	1.41±0. 05	0.61±0.07

PAPER 3:

Supplemental matherial

Figure S1. *An. stephensi* larvae mortality exposed to permethrin. Estimated means of logarithmically transformed mortality rate of each tested treatment. Both time of exposure and treatment were significant explanatory variables of the logarithmically transformed mortality rate (F=162.557, P<0.001, and F=5.381, P<0.01, respectively).

Dependent variable	Independent variables	F	df	P-value
Ln(mortality rate)	Time of exposure (continuous variable)	162.557	1	**<0.001*** **
	Treatment (categorical variable)	5.381	4	**<0.01** **



Paper list

Published papers:

De Marco L., Sassera D., Epis S., Mastrantonio V., Ferrari M., Ricci I., Comandatore F., Bandi C., Porretta D., Urbanelli S. The choreography of the chemical defensome response to insecticide stress: insights into the *Anopheles stephensi* transcriptome using RNA-Seq. Sci Rep 2017, 7:41312

Mastrantonio V.*, Ferrari M.*, Epis S., Negri A., Scuccimarra G., Montagna M., Favia G., Porretta D., Urbanelli S., Bandi C. Gene expression modulation of ABC transporter genes in response to permethrin in adults of the mosquito malaria vector *Anopheles stephensi*. Acta Trop 2017, 171:37

Porretta D., Epis S., Mastrantonio V., Ferrari M., Bellini R., Favia G., Urbanelli S. How heterogeneous is the involvement of ABC transporters against insecticides? Acta Trop 2016, 157:131

Manuscript in submission:

Ferrari M., Negri A., Romeo C., Varotto Boccazzi I., Nodari R., Habluetzel A., Molteni G., Corbett Y. ABC transporters are not involved in the detoxification of *Azadirachta indica* extracts in *Anopheles stephensi* larvae.
Manuscript in submission at Asian Pacific Journal of Tropical Disease

Manuscript in preparation:

Ferrari M., et al. ABCG4 silencing in *Anopheles stephensi* larvae increases insecticide effectiveness.